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Brassinosteroids control meristem size by promoting cell cycle progression in *Arabidopsis* roots

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SUMMARY

Brassinosteroids (BRs) play crucial roles in plant growth and development. Previous studies have shown that BRs promote cell elongation in vegetative organs in several plant species, but their contribution to meristem homeostasis remains unexplored. Our analyses report that both loss- and gain-of-function BR-related mutants in *Arabidopsis thaliana* have reduced meristem size, indicating that balanced BR signalling is needed for the optimal root growth. In the BR-insensitive *bri1-116* mutant, the expression pattern of the cell division markers *CYCB1;1*, *ICK2/KRP2* and *KNOLLE* revealed that a decreased mitotic activity accounts for the reduced meristem size; accordingly, this defect could be overcome by the overexpression of *CYCD3;1*. The activity of the quiescent centre (QC) was low in the short roots of *bri1-116*, as reported by cell type-specific markers and differentiation phenotypes of distal stem cells. Conversely, plants treated with the most active BR, brassinolide, or mutants with enhanced BR signalling, such as *bes1-d*, show a premature cell cycle exit that results in early differentiation of meristematic cells, which also negatively influence meristem size and overall root growth. In the stem cell niche, BRs promote the QC renewal and differentiation of distal stem cells. Together, our results provide evidence that BRs play a regulatory role in the control of cell-cycle progression and differentiation in the *Arabidopsis* root meristem.

KEY WORDS: Brassinosteroids, Root, Meristem, Cell division, Quiescent centre, Stem cells, Columella, Cell elongation

INTRODUCTION

Apical growth in both shoots and roots is a defining feature of vascular plants (Graham et al., 2000). Generative apices, established early in embryonic development, sustain polar growth and generate radial patterns of tissues along the body axes. Production of new cells is ensured by groups of stem cells located in the meristems. These cells have a low proliferative activity and divide asymmetrically. Although one of the daughter cells takes over the role of the stem cell, the other sets off into a proliferation and differentiation program that progresses as its lineage leaves the meristematic region. Thus, net growth is the outcome of a balance between cell proliferation, differentiation and elongation.

The primary root of *Arabidopsis thaliana* is a favourite developmental model because of its simple and stereotyped organization of cell types (Dolan et al., 1993; Wildwater et al., 2005; Sarkar et al., 2007). Root tips provide the most easily accessible group of stem cells in the plant body and have been extensively used to visualize the dynamics of cell division and elongation (Beemster and Baskin, 1998; Casamitjana-Martinez et al., 2003; Delo Ioio et al., 2007). These initials abut on the quiescent centre (QC), a group of three to four cells with very low proliferative activity that have been proposed to maintain the stem cell identity by means of short-range signals of a still unknown nature (Dolan et al., 1993; van den Berg et al., 1997; Aida et al., 2004). The position of the QC is defined by the overlap between

domains of high auxin levels at the root tip (Sabatini et al., 1999), highly expressed auxin-responsive members of the *PLETHORA* (*PLT*) family of transcription factors (Aida et al., 2004; Galinha et al., 2007) and the radial expression of the transcription factors *SCARECROW* (*SCR*) and *SHORT ROOT* (*SHR*) (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003).

Root growth is strongly inhibited by treatments with growth factors, indicating disturbances in the meristem activity. This observation was at the basis of many genetic screens that led to the identification of mutants impaired in the perception of growth regulators, such as auxins and brassinosteroids (BRs).

BRs are polyhydroxylated steroids that play crucial roles in plant cell growth and differentiation (Asami et al., 2000). The first mutant unable to respond to BRs was obtained by scoring roots that kept growing in the presence of an isomer of the physiologically most active BR, brassinolide (BL) (Clouse et al., 1996). These mutants carried a mutation in the *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) gene, which codes for a membrane-anchored leucine-rich repeat receptor-like kinase (LRR-RLK) (Li and Chory, 1997). Upon direct binding of BL to the extracellular domain (Kinoshita et al., 2005), *BRI1* heterodimerizes with its co-receptor *BRI1 ASSOCIATED RECEPTOR KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3* (*BAK1/SERK3*), another LRR-RLK (Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004). An intracellular phosphorylation relay involving at least two types of kinases (Tang et al., 2008; Wang et al., 2008) conveys the signal. Eventually, phosphorylation modulates the activity and stability of the *BRI1-EMS-SUPPRESSOR 1* (*BES1*) and *BRASSINAZOLE RESISTANT 1* (*BZR1*) genes (Li et al., 2002; Wang et al., 2002; Yin et al., 2002; Mora-García et al., 2004; Gampala et al., 2007). *BES1* and *BZR1* are members of a plant-specific family of basic-helix-loop-helix transcription factors that act as homo- or heterodimers (Yin et al., 2002; He et al., 2005; Li et al., 2008; Yu et al., 2008). Genes involved in cell wall loosening and in BR, auxin

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and ethylene synthesis or perception are among the main targets of this regulatory pathway. The gain-of-function *bes1-D* mutation results in an increased signalling output, as evidenced by the behaviour of BR-regulated genes in the mutant (Yin et al., 2002).

Originally discovered by their ability to stimulate stem growth at low concentrations, BRs are generally described as promoters of cell elongation. Indeed, BR-related mutants exhibit a short-root phenotype (Li et al., 1996; Müssig et al., 2003; Mouchel et al., 2006) and exogenous application of BRs appears to promote root growth at low concentrations, but to inhibit it at high concentrations (Roddick et al., 1993; Clouse et al., 1996; Müssig et al., 2003). BRs are seemingly also implicated in cell proliferation (Clouse et al., 1993); in fact, BL was reported to promote cell division in tobacco Bright-Yellow 2 cells (Hu et al., 2000; Nakaya et al., 2002; Miyazawa et al., 2003). However, the dwarfed leaf phenotype of some BR synthesis mutants seems to arise from reduced cell size rather than from decreased cell number (Szekeres et al., 1996).

Here, we investigated the role of BRs on root growth. Loss-of-function *bril-116* null mutants (Li and Chory, 1997) and gain-of-function *bes1-D* mutants (Yin et al., 2002) showed that a balance in BR signalling is required to maintain meristem size and overall root growth. The behaviour of the cell division markers *pCYCB1;1*, *pICK2/KRP2* and *KNOLLE*, together with the recapitulation of the *bril-116* meristem defects by overexpression of *CYCD3;1* revealed that BR signalling is needed for a normal cell cycle progression. Additionally, our results uncover the importance of BRs in controlling the activity of the stem cell niches in plants.

MATERIALS AND METHODS

Plant lines and growth conditions

Arabidopsis thaliana (L.) Heyhn. was in Columbia-0 (Col-0) background. To avoid ecotype variability, the *bes1-D* mutant, originally in Enkheim-2 (En-2) background (Yin et al., 2002), was introgressed into the Col-0 ecotype. The *BR11*-null mutant *bril-116* (Li and Chory, 1997) and a transgenic line overexpressing *BR11-GFP* (Friedrichsen et al., 2000; Wang et al., 2001) were used as examples of insensitivity to BRs and of enhanced BR signalling, respectively. Other lines used were: *pCYCB1;1:GUS* (Colón-Carmona et al., 1999), *pICK2/KRP2:GUS* (De Veylder et al., 2001), a *CYCD3;1*-overexpressing line (Riou-Khamlichi et al., 1999), *CYCB1;1:GFP* (Ubeda-Tomás et al., 2009), *KNOLLE-GFP* (Volker et al., 2001), *pWOX5:GFP* (Sarkar et al., 2007), *QC25:CFP*, *QC46:GUS*, *QC142:GUS* and *QC184:GUS*, *pSCR:GFP* (Sabatini et al., 1999), *pAGL42:GFP* (Nawy et al., 2005), *scr-4* (Fukaki et al., 1998), and *wox5-1* (Sarkar et al., 2007). Seeds were surface sterilized in 35% sodium hypochlorite, vernalized for 72 hours at 4°C in darkness and grown on vertically oriented plates containing 1× Murashige and Skoog (MS) salt mixture, 1% sucrose and 0.8% agar in the absence or presence of different concentrations of BL (C₂₈H₄₈O₆; Wako, Osaka, Japan). For short-term treatments plants were treated with 1 μM BL for 4 hours, 8 hours and 24 hours. Plates were incubated at 22°C and 70% humidity under long-day conditions (16 hours light/8 hours dark).

Confocal microscopy

A FV 1000 confocal microscope (Olympus, Tokyo, Japan) was used throughout the study. Roots were stained in 10 μg/ml propidium iodide (PI) for 2–5 minutes, rinsed, mounted in dH₂O, and visualized after excitation by a Kr/Ar 488-nm laser line. PI and GFP were detected with a band-pass 570–670 nm filter and 500–545 nm filter, respectively. For the yellow and cyan fluorescent proteins (YFP and CFP)-tagged reporters, the excitation wavelengths were 488 nm and 405 nm, and fluorescence was collected in the ranges of 493–536 nm (rendered in green) and 460–500 nm, respectively. Starch granules in columella cells were visualized by a modified Pseudo-Schiff (mPS)-PI staining method (Truernit et al., 2008). Dividing cells in *CYCB1;1:GFP* and *KNOLLE:GFP* plants were manually counted from confocal stacks. Images were processed with the Olympus FV software and assembled with Photoshop CS (Adobe Systems, San Jose, CA, USA).

Statistical analysis of root measurements

Root length, cell length and number of meristem cells in *Arabidopsis* roots were assessed in at least three independent experiments. Roots were scanned and root length measured with ImageJ software (<http://rsb.info.nih.gov/ij/>). For comparisons, Student's *t*-tests were performed in all cases (see Table S1 in the supplementary material).

Cell length was measured along single epidermal cell files. The number of epidermal cells in individual cell files was used to gauge the meristem size. The meristematic zone was defined as the region of isodiametric cells from the QC up to the cell that was twice the length of the immediately preceding cell. For dose-response assays, the average distance from the QC to each individual cell was computed as a function of the cell number. Linear regressions were applied to the average distance from the QC as a function of cell number for both the meristematic and the elongation zone. Consequently, the slope of the linear regions of the curves reflected the average cell length in each zone.

Quantification of cell division in β-glucuronidase (GUS)-stained roots

For β-glucuronidase (GUS) detection, 6-day-old seedlings were immersed on ice-cold 90% (v/v) acetone, incubated 20 minutes on ice, rinsed in dH₂O, infiltrated with 100 mM sodium phosphate buffer (pH 7.2), 10 mM sodium EDTA, 0.1% Triton X-100, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Xgluc; Duchefa, Haarlem, The Netherlands), 10 mM potassium ferrocyanide and potassium ferricyanide, and incubated at 37°C for 3 hours. Samples were rinsed three times in dH₂O, treated with 70% ethanol, and cleared in chloral hydrate. Stained roots were visualized with an AxioPhot (Zeiss, Jena, Germany) microscope. Digital images were acquired with a DP70 (Olympus) camera. The number of GUS-stained cells in the roots of genotypes carrying *pCYCB1;1:GUS* was recorded with an AxioPhot (Zeiss) microscope.

Immunohistochemistry

Whole-mount immunolocalization in *Arabidopsis* roots was carried out as described (Sauer et al., 2006) with minor modifications. For WOX5:GFP, polyclonal anti-GFP IgG and Alexa Fluor 488 conjugate (1:200) (Invitrogen, Carlsbad, CA, USA) were used. To detect cell plates formed in dividing cells, we used anti-KNOLLE primary antibodies (1:1000) (Volker et al., 2001) and Alexa555-coupled anti-rabbit secondary antibody (Invitrogen). Slides were mounted in 4',6-diamidino-2-phenylindole (DAPI) Vectashield medium (Vector Laboratories, Burlingame, CA, USA) and placed at 4°C. DAPI and Alexa488 signals were acquired sequentially into separate channels: for Alexa555, samples were excited at 559 nm and fluorescence detected in the range 550–600 nm; for DAPI, the excitation wavelength was 405 nm and fluorescence collected in the range 425–475 nm.

Quantitative real-time PCR

Total RNA from root tips was extracted with Plant RNeasy Mini Kit (Qiagen, Hilden, Germany), DNA contaminations were removed with the DNA-free Kit (Ambion, Austin, TX, USA) and cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen), all according to the manufacturer's instructions. Oligonucleotides were designed with the Primer Express Software (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). At3g24250 forward 5'-CTTGGTGGTTTGGGTG-GTG-3' and reverse 5'-ACGATAAGAGAAACACAGAAAC-3'; At3g11260 forward 5'-TGATCTGTTTCGAGCCGGTC-3' and reverse 5'-AAACATTCTTGCTCTCTATCTTGCC-3'.

PCR products were detected with the SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). LightCycler480 software 1.5.0 release (Roche Diagnostics) was used to calculate relative change in expression levels, with three technical replicates. Melting curves analyses at the end of the process and 'no template controls' were carried out to ensure product-specific amplification without primer-dimer artifacts. To evaluate genomic DNA contamination, a control reaction was run without reverse transcriptase. The expression of At3g24250 was used as an endogenous control under every condition.

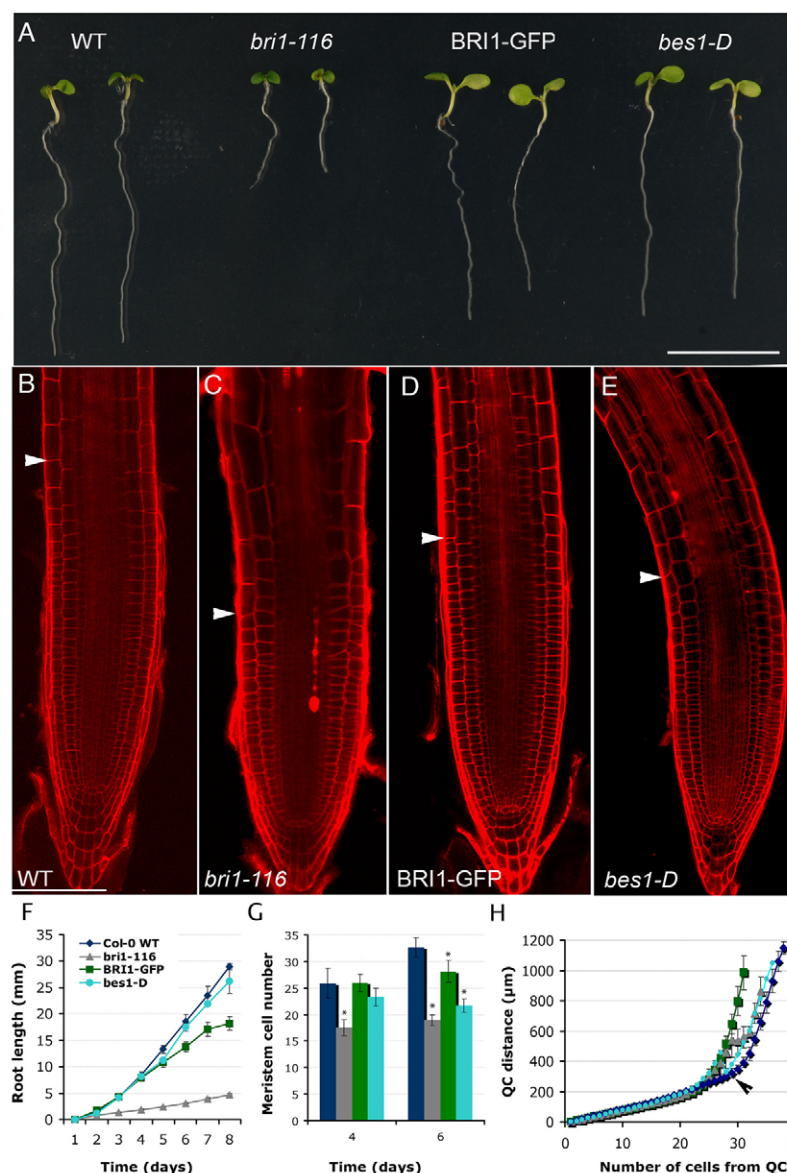


Fig. 1. Alterations in BRs signalling result in smaller root apical meristems. (A) Phenotype of 6-day-old wild-type Col-0 (WT), *bri1-116*, a transgenic line overexpressing *BRI1-GFP* (Friedrichsen et al., 2000; Wang et al., 2001) and *bes1-D* seedlings (Yin et al., 2002). Scale bar: 10 mm. (B-E) Confocal images of 6-day-old wild-type (B), *bri1-116* (C), *BRI1-GFP* (D) and *bes1-D* (E) roots stained with PI. Arrows indicate the boundary between the proximal meristem and the elongation zone of the root. Scale bars: 100 μm. (F) Root-length measurements of *bri1-116*, *BRI1-GFP* and *bes1-D* seedlings compared with the wild type (for statistical analysis, see Table S1 in the supplementary material). Values represent the mean of 80 measurements \pm s.d. (G) Meristem cell number in *bri1-116*, *BRI1-GFP* and *bes1-D* seedlings compared with the Col-0 (wild type) at 4 or 6 days after germination (asterisks indicate significant differences from Col-0 for each day) (for statistical analysis, see Table S1 in supplementary material). Values represent the mean of 34 measurements \pm s.d. (H) Spatial profile of epidermal QC distance (μm) versus number of cells from the QC of 6-day-old *bri1-116*, *BRI1-GFP*, *bes1-D* and wild-type roots. Epidermal cells were counted from the QC up to the appearance of the first root hair. All BR-related mutants analysed exhibited a premature exit from the meristem zone (black arrowhead) and early entry into the differentiation zone (grey arrowhead). Values represent the mean of 5 measurements \pm s.d.

RESULTS

Optimal BR signalling is required to control root meristem size

Root growth is determined by the balance between cell division and cell elongation (Beemster and Baskin, 1998). To assess the role of BRs in root growth, we first compared the root length of mutants with altered BR signalling by using *bri1-116* mutants (Li and Chory, 1997), transgenic lines overexpressing a green fluorescent protein (GFP)-tagged version of *BRI1* (Friedrichsen et al., 2000; Wang et al., 2001) and *bes1-D* plants carrying a gain-of-function mutation that enhances BR signalling (Yin et al., 2002). A general trend was observed for all BR-related mutants that displayed shorter roots than those of the wild type Col-0 (Fig. 1A,F).

Next, we studied the contribution of BRs to the elongation of root cells. Root epidermal cells in the differentiation zone, where cells have reached their final size of *BRI1-GFP*-overexpressing plants [216 ± 7 μm (s.d.), $P < 0.001$] and *bes1-D* [237 ± 3 μm (s.d.), $P < 0.001$] were longer than those of the wild type [139 ± 2 μm (s.d.)], whereas *bri1-116* mutant cells failed to elongate properly [91 ± 8 μm (s.d.), $P < 0.001$] (see Fig. S2 in the supplementary

material). These results are in agreement with previous observations showing that BRs induce cell elongation in the root (Szekeres et al., 1996; Müssig et al., 2003; Mouchel et al., 2006).

We also estimated the contribution of cell division to meristem size by counting the number of isodiametric epidermal cells. This number was significantly reduced in *bri1-116* mutants [19 ± 1 (s.d.), $P < 0.001$] (Fig. 1C,G) compared with that in the wild type [33 ± 2 (s.d.)] (Fig. 1B,G). In addition, the short roots of *BRI1-GFP* plants had a slightly reduced number of meristematic cells [28 ± 2 (s.d.), $P < 0.001$] (Fig. 1D,G). This reduction was more pronounced in the gain-of-function *bes1-D* mutants [22 ± 1 (s.d.), $P < 0.001$] cells (Fig. 1E,G,H). The reduced number of meristematic cells in both loss- and gain-of-function mutants reveals the importance of a balanced BR signalling in the control of the meristem size.

BR effects on root meristem size are dose dependent

To study the effects of BRs on root growth, we treated wild-type Col-0 seedlings with BL concentrations in the range reported for the direct binding of BL to the *BRI1*-like receptor family (Kinoshita

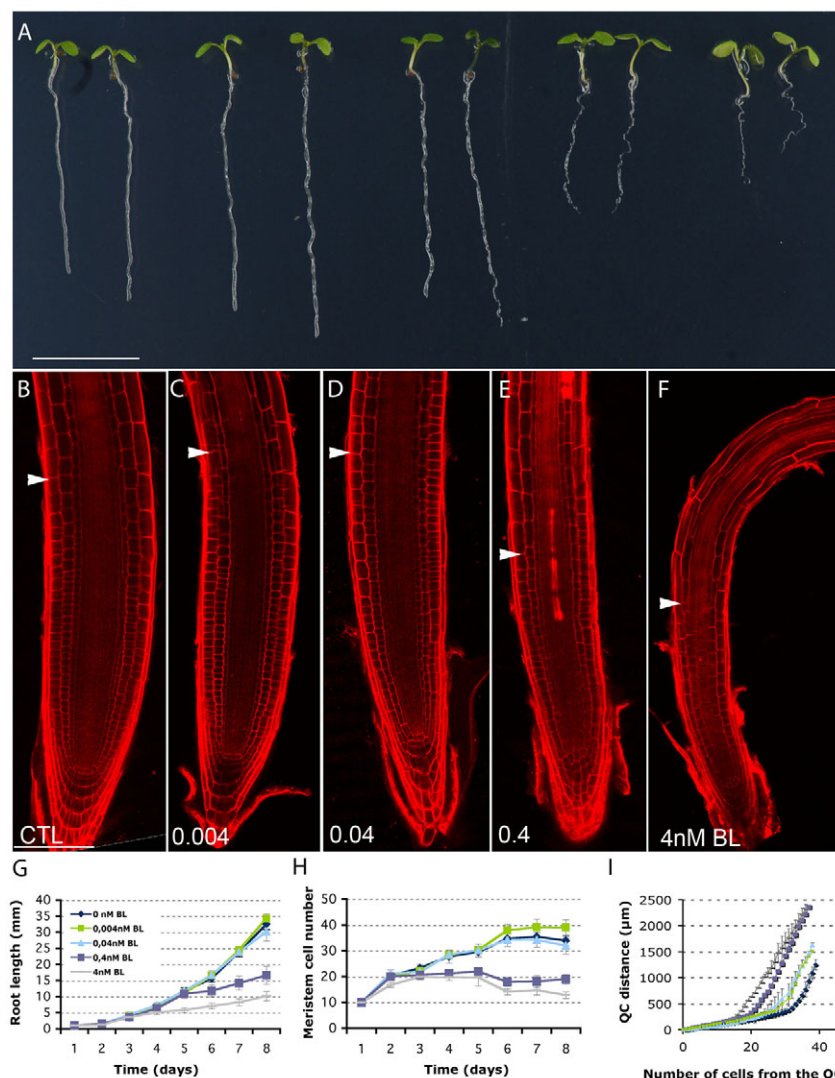


Fig. 2. Dose-dependent effects of BRs in root growth and meristem size. (A) Phenotype of 6-day-old wild-type *Col-0* plants from left to right control (CTL) and treated with 0.004 nM, 0.04 nM, 0.4 nM and 4 nM BL. Scale bars: 10 mm.

(B-F) Effect of exogenously applied BL on root growth. Images illustrate longitudinal median confocal images of 6-day-old primary *Col-0* roots treated with the indicated amounts of BL. Arrows indicate the boundary between the proximal meristem and the elongation zone of the root. Scale bar: 100 μ m. **(G)** Root length of *Col-0* seedlings grown in increasing BL concentrations. Values represent the mean of 30 measurements \pm s.d. Concentrations of at least 0.4 nM BL were found to be sufficient to repress root growth (for statistical analysis, see Table S1 in supplementary material). **(H)** Meristem cell number of *Col-0* roots grown in increasing BL concentrations for 8 days after germination. Values represent the mean of 80 measurements \pm s.d. **(I)** Spatial profile of cell elongation in 6-day-old *Col-0* roots treated with different BL concentrations. Epidermal cells were counted from the QC up to the appearance of the first root hair. Values represent the mean of 5 measurements \pm s.d.

et al., 2005). BL promoted root growth only at very low concentrations (≤ 0.04 nM, $P < 0.05$) in 6-day-old seedlings (Fig. 2A,G) (see Table S1 in the supplementary material) (Müssig et al., 2003); BL concentrations higher than 0.04 nM inhibited root growth and meristem size (Fig. 2B-F,G,H; $P < 0.001$). BR-induced root shortening was coupled to a reduction in meristem size rather than in cell elongation (Fig. 2G,H; $P < 0.001$). The number of meristematic cells in 6-day-old roots treated with 4 nM BL was reduced $\sim 60\%$ when compared with untreated roots (Fig. 2B,F,H; $P < 0.001$). A dose-response analysis revealed that BRs promoted the exit of cells from the meristematic region, and indicates that BRs promote cell elongation at the meristematic zone of the root (Fig. 2I). These results concur with our genetic analysis and support that BR signalling is required to maintain normal root growth rates through the control of the root meristem size.

BRs are required for proper cell-cycle progression in the root meristem

To investigate why altered BR signalling was detrimental for the root meristem size, we studied the effects of BRs on the cell cycle by analyzing the expression of the D-box *pCYCB1;1::GUS* reporter (Colón-Carmona et al., 1999) in BR-related mutants and in BL-treated plants. *pCYCB1;1::GUS* allows the visualization of cells at

the G2-M phase of the cell cycle. The number of GUS-stained cells was significantly reduced in BL-treated plants [7 ± 2 (s.d.), $P < 0.001$] (Fig. 3B) and in *bes1-D* mutants [6 ± 1 (s.d.), $P < 0.001$] (Fig. 3C) when compared with the wild type [34 ± 5 (s.d.)] (Fig. 3A,E). BL-treated plants exhibited a dose-dependent reduction of GUS-positive cells; similar results were obtained with the *CYCB1;1::GFP* reporter (see Fig. S3 in supplementary material). In *bril-116* mutants, a slight increase in the expression levels was observed for the *pCYCB1;1::GUS* reporter (Fig. 3D,E) ($P < 0.05$), whereas *bril-116::CYCB1;1::GFP* roots showed a reduction in total GFP-marked cells (see Fig. S3 in the supplementary material; $P < 0.001$). These differences may be attributed to the fact that *CYCB1;1::GFP* is mainly expressed in peripheral cell layers, whereas *pCYCB1;1::GUS* is expressed in all root tissues (see Fig. S3 in the supplementary material).

We reasoned that the alterations in *CYCB1;1* expression in the *bril-116* background could reflect a slower progression and/or a delayed G2/M transition of the cell cycle. To corroborate this hypothesis, a number of independent experiments were carried out. The cytokinesis-specific syntaxin KNOLLE required for the M-phase progression was used as a marker for cell division planes (Volker et al., 2001). Using anti-KNOLLE antibodies, the number of cell plates was significantly reduced in 6-day-old plants treated

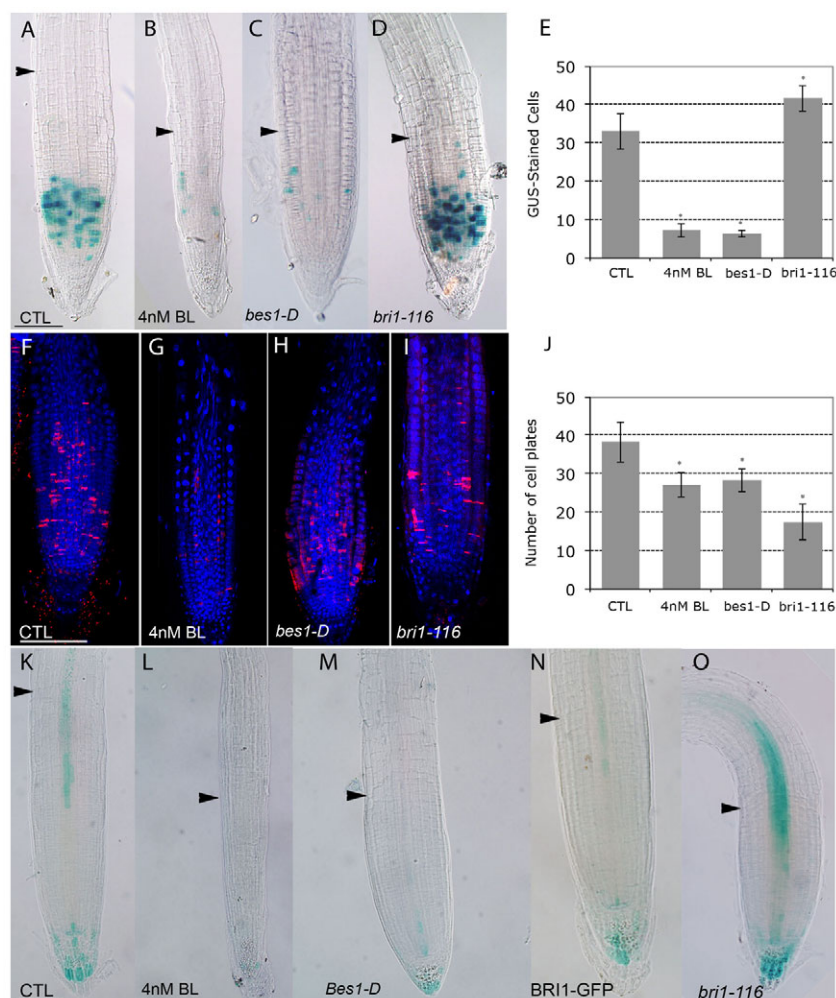


Fig. 3. Regulation of cell-cycle progression by BRI1 signalling in the root meristem.

(A–D) *pCYCB1;1:GUS* expression in 6-day-old roots. (A) *pCYCB1;1:GUS* (control; CTL), (B) *pCYCB1;1:GUS* plants treated with 4 nM BL, (C) F3 roots of *bes1-D*; *pCYCB1;1:GUS* homozygous plants and (D) F3 roots of *bri1-116*; *pCYCB1;1:GUS* homozygous plants. (E) Number of GUS-stained cells in the meristem of *pCYCB1;1:GUS* ($P < 0.001$) not treated or treated with 4 nM BL, in *bes1-D*; *pCYCB1;1:GUS* ($P < 0.001$) and *bri1-116*; *pCYCB1;1:GUS* ($P < 0.05$) seedlings. Asterisks indicate a statistically significant difference from the wild-type control. Values represent the mean of 25 measurements \pm s.d. (F–I) Whole-mount immunofluorescence with anti-KNOLLE antibodies (Volker et al., 2001) in untreated (F) or 4-nM BL-treated Col-0 (G), and in *bes1-D* (H) and *bri1-116* (I) roots. Nuclei were counterstained with DAPI (blue). Scale bar: 100 μ m. (J) Reduced number of cell plates in 4-nM BL-treated Col-0 ($P < 0.01$), and in *bes1-D* ($P < 0.05$) and *bri1-116* ($P < 0.001$) roots. Asterisks indicate a statistically significant difference from the untreated Col-0 control. Values represent the mean of 10 measurements \pm s.d. (K–O) *pICK2/KRP2:GUS* (CTL) expression in 6-day-old roots untreated (K), treated with 4 nM BL (L), and *bes1-D*; *pICK2/KRP2:GUS* (M), *BRI1-GFP*; *pICK2/KRP2:GUS* (N) and *bri1-116*; *pICK2/KRP2:GUS* homozygous plants (O). Arrowheads indicate the boundary between the proximal meristem and the elongation zone of the root. Scale bar: 100 μ m.

with 4 nM BL (Fig. 3G) ($P < 0.01$), and in *bes1-D* (Fig. 3H) ($P < 0.05$) and *bri1-116* roots (Fig. 3I) ($P < 0.001$) compared with the wild type (Fig. 3F,J). Similar results were obtained with a translational *KNOLLE:GFP* line (see Fig. S3 in the supplementary material), further demonstrating that both loss- and gain-of-function BR-related mutants exhibit cell division defects that negatively impact on the meristem size.

Next, a reporter for the plant-specific cell cycle inhibitor *pICK2/KRP2:GUS* (De Veylder et al., 2001) was analyzed in plants with altered BR signalling. The expression of *pICK2/KRP2:GUS* was dramatically reduced in plants with increased BR levels (i.e. 4 nM BL-treated plants) (Fig. 3K,L), *bes1-D* (Fig. 3M) and in *BRI1-GFP*-overexpressing plants (Fig. 3N), whereas it was increased in *bri1-116* roots (Fig. 3O).

Because the accumulation of *pICK2/KRP2:GUS* in the *bri1-116* roots could reflect an arrest in cell division, we investigated whether an increase in the mitotic activity could reverse the cell-cycle defects of this mutant by overexpressing *CyclinD3;1* (*CYCD3;1OE*) (Riou-Khamlichy et al., 1999). The number of meristematic cells in *bri1-116*; *CYCD3;1OE* plants was indeed higher than in the *bri1-116* mutants and similar to that of wild-type or *CYCD3;1OE* plants (Fig. 4A–E). Moreover, like *CYCD3;1OE* plants that have a supernumerary layer of columella cells (Riou-Khamlichy et al., 1999) (Fig. 4G), *bri1-116*; *CYCD3;1OE* plants also had an increased number of columella cells and rescued the lack of cell division of *bri1-116* (compare Fig. 4F,H with 4I). Cell-

cycle progression defects observed in a *BRI1*-deficient background can thus be restored by stimulating cell divisions. These results indicate that a balanced BR signalling is required for a normal progression of the cell cycle in the root meristem, contributing to the regulation of meristem size and root growth.

BRI1 signalling controls QC identity

Root cell proliferation is initiated within a population of stem cells surrounding the QC (Scheres, 2007). Thus, we investigated whether the reduction in root meristem size observed in BR-related mutants could derive from defects in the stem cell niche. Significant BL concentration-dependent alterations occurred in the expression of the QC-localized markers *pWUSCHEL-RELATED HOMEODOMAIN 5* (*WOX5*):*GFP*, *pAGL42:GFP*, *pSCR:GFP* and four independent promoter-trap lines (*QC25:CFP*, *QC142:GUS*, *QC46:GUS* and *QC184:GUS*) (Sabatini et al., 1999; Nawy et al., 2005; Sarkar et al., 2007) (Fig. 5; see Figs S4 and S5 in the supplementary material). Specifically, the expression of *pWOX5:GFP* (Fig. 5A–G), *pAGL42:GFP* (Fig. 5H,I), *pSCR:GFP* (Fig. 5J,K), *QC25:CFP* (Fig. 5L,M) and *QC142:GUS* (Fig. 5N,O) was enhanced by continuous application of 4 nM BL. Conversely, the expression of *QC46:GUS* (Fig. 5P,Q) and *QC184:GUS* (Fig. 5R,S) was reduced, whereas the reporter fusions for the three *PLETHORA* (*PLT*) family members (Galinha et al., 2007) did not show significant changes in response to the BL treatment (see Fig. S8 in the supplementary material).

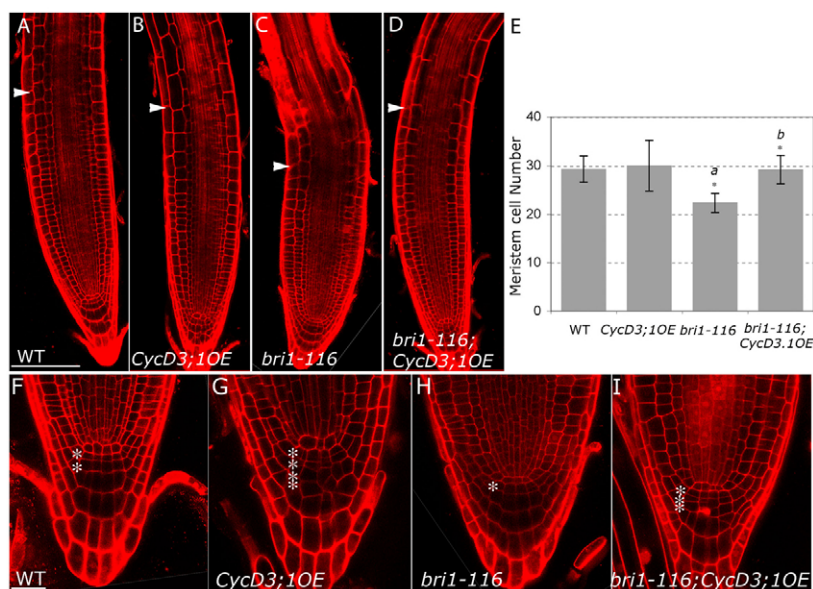


Fig. 4. Rescue of the phenotype of *bri1-116* mutants in the root meristem and columella cells by overexpression of *CYCD3;1*. (A–D) Phenotype of 6-day-old Col-0 (wild type) (A), *CYCD3;1OE* (B), *bri1-116* (C) and double *bri1-116;CYCD3;1OE* (D) roots.

Arrowheads indicate the boundary between the meristematic and elongation zones of the root. (E) Meristem cell number in the roots of 6-day-old *CYCD3;1OE*, *bri1-116* and *bri1-116;CYCD3;1OE* plants compared with the wild type Col-0. Asterisk indicates significant differences compared with the wild type ($P < 0.01$) (a) and with *bri1-116* ($P < 0.01$) (b). Values represent the mean of 30 measurements \pm s.d.

(F–I) Confocal images of 6-day-old Col-0 (F), *CYCD3;1OE* (G), *bri1-116* (H) and *bri1-116;CYCD3;1OE* (I) primary roots stained with PI. Asterisks indicate the layers of CSCs. Scale bar: 100 μ m.

An increased number of cells expressing *pWOX5:GFP* occurred already after 4 hours of BL treatment (Fig. 6A–H). Genetic crosses of *pWOX5:GFP* with BR-related mutants revealed that *bri1-116* roots had a reduced expression of *pWOX5:GFP* in the absence of BRI1 signalling (Fig. 6I,J,M,N). Conversely, the number of cells expressing the *WOX5* marker had increased in *BRI1-GFP* plants (Fig. 6K,O,Q), resembling short-term BL treatments (Fig. 6J–M). This effect was even stronger in a *bes1-D* background, where the *WOX5* expression domain appeared to double in size (Fig. 6L,P,Q), indicating that the *WOX5* expression is controlled downstream of the core-BR signalling components BRI1 and BES1. Strikingly, exogenous application of the auxin 1-naphthalene acetic acid (NAA) or the inhibitor of auxin transport *N*-1-naphthylphthalamic acid (NPA) failed to mimic the effect of BRs on the expression of *WOX5* (see Fig. S6 in the supplementary material), showing that the effects of BRs in the QC are not associated with alterations in auxin levels or distribution in the root apex.

The effect of BL was also observed by whole-mount immunolocalization of *pWOX5:GFP* (compare Fig. 7A–C with 7D–F). The identity of the new cells expressing *WOX5* was investigated by analyzing the expression of *pSCR:GFP* in the QC (Sabatini et al., 2003) (Fig. 7G–J). Both *bes1-D* mutants and BL-treated plants expressed *pSCR:GFP* in additional cells compared with the wild type (Fig. 7L,J versus 7G). However, the *WOX5* expression domain was larger than that of *SCR*, suggesting that BRs are able to promote the expression of *WOX5* independently of *SCR*. These results address a role for the BRI1-dependent signalling pathway in the control of the QC identity.

BRs promote the differentiation of distal columella stem cells

To study the functional relevance of the misexpression of QC-associated genes in BR-related mutants, we investigated whether columella stem cells (CSCs) undergo abnormal differentiation visualizing starch granules accumulation (Truernit et al., 2008). In agreement with previous results (Stahl and Simon, 2009), 80% of the wild-type plants had a single layer of CSCs and 18% two layers adjacent to the QC (Fig. 7K,S), whereas 100% of the *bri1-116* roots had a single layer of CSCs ($n=60$) (Fig. 7L,S). Although starch granules are absent from wild-type CSCs and only appear in

differentiating columella cells, starch granules occurred in cells at the position of CSCs in *bes1-D* roots (Fig. 7M,S) ($n=45$) and in plants treated with 4 nM BL (Fig. 7N,S) ($n=25$). These results show that BRs promote the differentiation of distal stem cells at the root apex (Fig. 7T), revealing the importance of BRs in the control of root stem cell dynamics. Additional support for this statement is provided by an increase in differentiated columella cells in *CYCD3;1OE* plants treated with 4 nM BL (see Fig. S3 in the supplementary material).

To unveil the genetic relationship between BR signalling and key components of CSC differentiation, the *bri1-116* mutation was combined with the null mutations *scr-4* (Fig. 7O) (Fukaki et al., 1998) and *wox5-1* (Fig. 7Q) (Sarkar et al., 2007), and with double *plt1;plt2* mutants (Aida et al., 2004). Six-day-old double *bri1-116;scr-4* and *bri1-116;wox5-1* mutant roots showed CSC phenotypes identical to those of the *scr-4* (Fig. 7O,P,S) and *wox5-1* (Fig. 7Q–S) single mutants, and *wox5-1* mutants were insensitive to BR for the QC proliferation phenotype (Fig. S7 in the supplementary material). By contrast, the additive effects in the *bri1-116;plt1;plt2* triple mutants (Fig. S8 in the supplementary material) suggest that these genes might act independently in CSC differentiation. These results confirm our expression analyses showing that *SCR* and *WOX5* act downstream of *BRI1* in CSC differentiation and reveal the importance of BRs in the control of root stem cell dynamics (Fig. 8).

DISCUSSION

The role of BRs in promoting cell elongation is not sufficient to explain the short root phenotypes observed in both gain- and loss-of-function BR-related mutants. We show that balanced levels of the BRI1 receptor and the downstream regulator BES1 are needed to maintain normal cell division activities in the root meristem. We propose that BRs act on the root stem cell niche both triggering the self-renewing competence of QC cells and controlling the cell cycle progression necessary for maintaining the meristem size in the daughter cells. Altered BR signalling provokes changes in the cell cycle progression, leading to defects in meristem cell proliferation and differentiation that ultimately result in short roots.

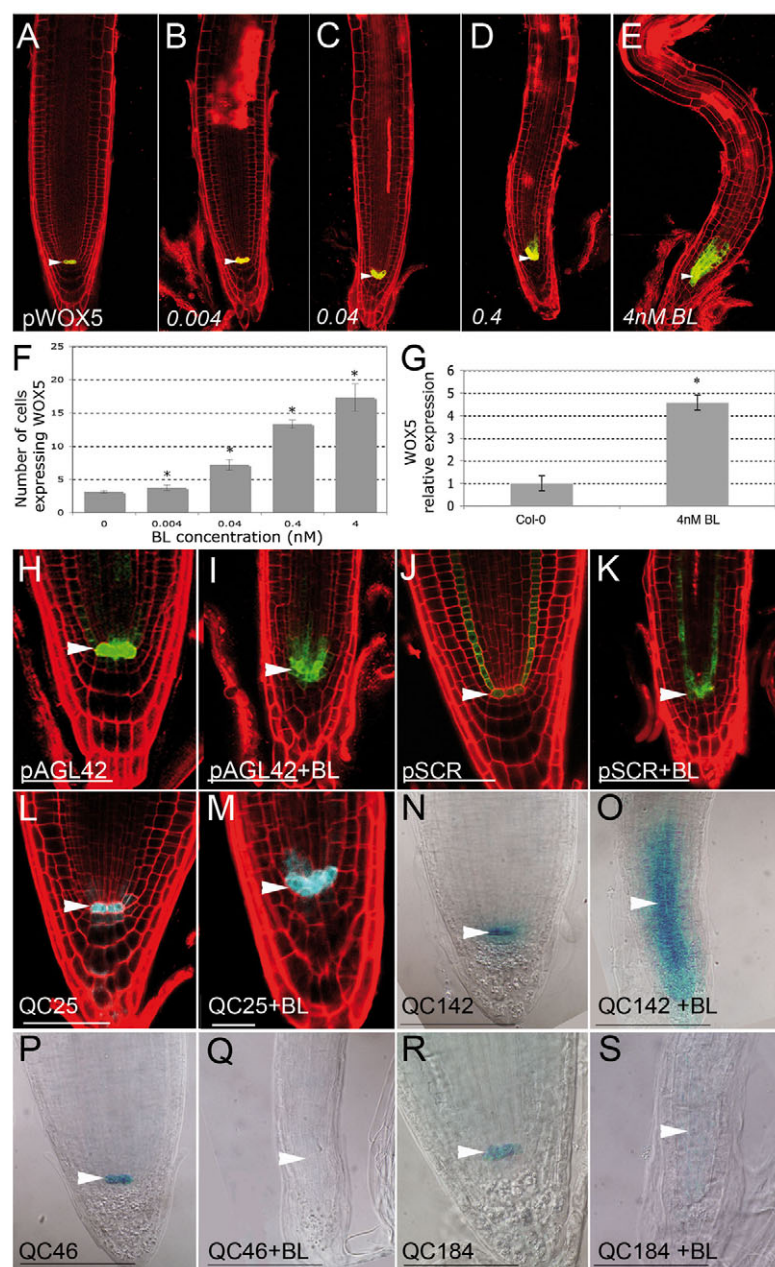


Fig. 5. Regulation of the expression of QC-specific markers by BRs. (A-E) Confocal images of primary roots expressing *pWOX5:GFP* in the QC cells. Six-day-old roots untreated (A) or treated with 0.004 nM BL (B), 0.04 nM BL (C), 0.4 nM BL (D) and 4 nM BL (E). Roots were counterstained with PI. (F) Number of cells expressing the *pWOX5:GFP* marker in roots untreated or treated with 0.004 nM BL ($P < 0.05$), 0.04 nM BL ($P < 0.001$), 0.4 nM BL ($P < 0.01$) and 4 nM BL ($P < 0.01$). Values represent the mean of 15 measurements \pm s.d. Asterisks indicate a statistically significant difference from the wild-type control. (G) Quantitative reverse-transcription PCR levels of the *WOX5* gene in wild-type Col-0 and plants grown for 6 days with 4 nM BL ($P < 0.001$). Values represent the mean of 3 measurements \pm s.d. Asterisks indicate a statistically significant difference from the wild-type control. (H-S) Expression patterns of QC markers *pAGL42:GFP* (H,I) and *pSCR:GFP* (J,K), and of promoter-trap lines *QC25:CFP* (L,M), *QC142:GUS* (N,O), *QC46:GUS* (P,Q) and *QC184:GUS* (R,S) in 6-day-old roots untreated or treated with 4 nM BL. Arrowheads indicate QC cells. Scale bars: 100 μ m.

BRs restrain root growth by controlling the normal cell cycle progression in the root meristem

To date, the contribution of BRs to plant growth has been mostly based on their ability to promote cell expansion of shoot organs (Mandava, 1988). The dwarfed phenotype of BR-deficient mutants has so far been interpreted as a failure to carry out proper cell elongation (Clouse et al., 1996; Li et al., 1996; Yin et al., 2002). Indeed, the reduced cell size observed in the short *bri1-116* roots, defective in the principal BR receptor, supports the notion that BRs play an important role in cell elongation during primary root growth. Our data showing that plants with increased BR signalling also exhibit reduced root growth indicate that BRI1-mediated promotion of cell elongation (Müssig et al., 2002; De Grauwe et al., 2005; Tong et al., 2009) is not sufficient to produce long roots. The reduction in cell numbers in the meristem of BR-related mutants implies the existence of additional mechanisms whereby BRs regulate root growth, such as cell division and differentiation.

The higher effect in root growth of BL compared with that observed in *bes1-D* mutation may be attributed to cell-type and timing-of-expression specific effects, as similar effects have also been reported for other developmental phenotypes (Yin et al., 2002; Ibañez et al., 2009). Thus, it is expected that the dimension of BR responses caused by the exogenous ligand may be wider than those of the *bes1-D* downstream component.

The role of BRs in cell proliferation has remained controversial (Clouse et al., 1993; Hu et al., 2000). We have recently shown that BRs contribute to vascular patterning by triggering provascular cell divisions in *Arabidopsis* shoots (Ibañez et al., 2009). How BRs precisely control cell division is not yet known, but we demonstrate that BR signalling components are involved in the promotion of cell cycle progression in the root meristem.

Whether BR signalling components regulate different cell cycle phases remains an open question. On the one hand, in *bri1-116* mutants, the altered expression of the *CYCB1;1* reporter, the

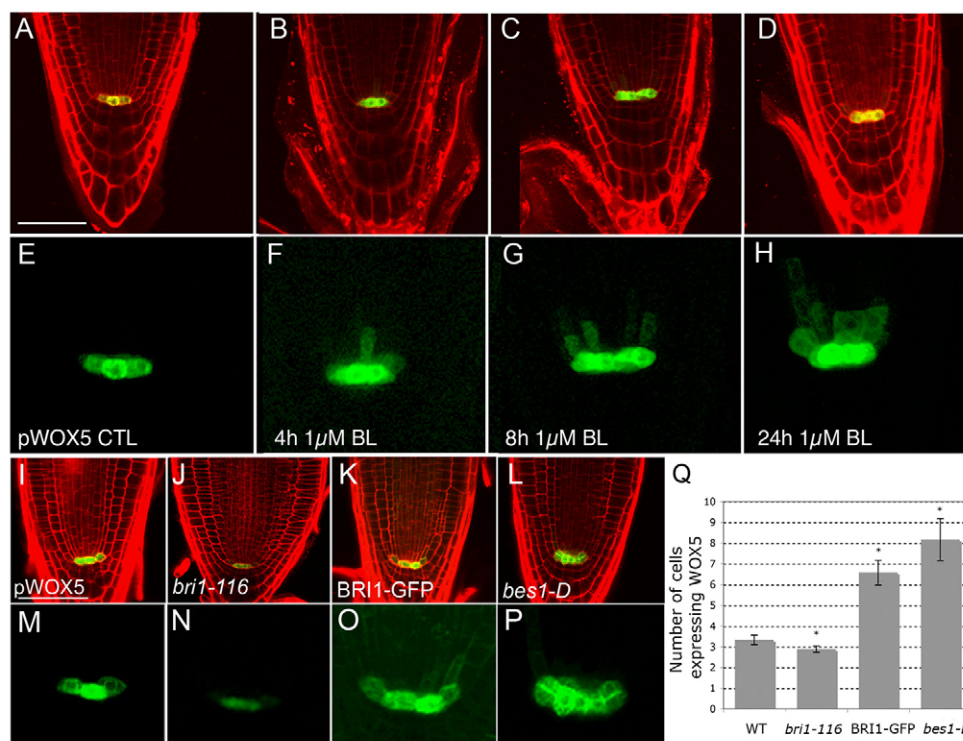


Fig. 6. Expression analysis of *pWOX5:GFP* in response to short BL treatments and in BR-related mutant backgrounds.

(A–H) Longitudinal view of 6-day-old root meristems expressing *pWOX5:GFP* without treatment (A, E) and treated with 1 μ M BL for 4 hours (B, F), 8 hours (C, G) and 24 hours (D, H). Roots were counterstained with PI. (I–P) *pWOX5:GFP* expression in wild-type (I, M), *bri1-116* (J, N), *BRI1-GFP* (K, O) and *bes1-D* (L, P) 6-day-old roots. Scale bar: 50 μ m. (Q) Number of cells expressing the *pWOX5:GFP* marker in the different mutant backgrounds *bri1-116* ($P < 0.05$), *BRI1-GFP* ($P < 0.001$) and *bes1-D* ($P < 0.001$) compared with the control line (WT) ($n = 9$). Asterisk indicates a statistically significant difference between mutants and the wild type. Values represent the mean of 12 measurements \pm s.d.

decreased number of cell plates and the accumulation of the cell-cycle inhibitor *ICK2/KRP2* (De Veylder et al., 2001) reflect a reduced cell division activity. The overexpression of *CYCD3;1* (Riou-Khamlichi et al., 1999) alleviated the meristem defects of a *bri1*-deficient mutant, unequivocally coupling BR-mediated root growth to cell cycle progression in the meristem (Fig. 8A). On the other hand, the mitotic activity in the root meristem was also reduced in the gain-of-function BR-related mutants. BRs have been shown to promote *CYCD3;1* expression (Hu et al., 2000). Increased BRs signalling triggered the differentiation of the supernumerary columella cells in BL-treated *CYCD3;1OE* plants (see Fig. S3X, Y in the supplementary material) and led to the appearance of root hairs near the root tip, indicating a premature exit from the cell cycle and a faster transition to cell differentiation. The longer cells observed in plants with increased BRs signalling could be part of a compensation mechanism (De Veylder et al., 2001): in the *bes1-D* mutant, the short meristem co-exists with elongated cells, producing roots only slightly shorter than those of the wild type.

Our study reveals that BR signalling regulates the balance between cell division and differentiation necessary to maintain meristem size during root growth. Whether BR signalling components regulate different cell cycle phases remains an unresolved issue.

BR signalling control of stem cell renewal in the primary root

The QC acts as a organized of root meristematic cells (Dolan et al., 1993; van den Berg et al., 1997; Aida et al., 2004) but also has a low proliferation rate (Dolan et al., 1993; van den Berg et al., 1995) that might indicate that it can be a source for new stem cells. The fate of stem cells surrounding the QC can be used as a readout of its organizing activity. By looking at the expression of QC-specific markers and the differentiation of CSCs, we found that BRs modulate the activity of the stem cell niche at the root apex (Fig. 8C). The reduced expression of the QC identity markers

pSCR:GFP and *pWOX5:GFP* in *bri1-116* mutants, their ectopic expression in plants with increased BR-signalling and the phenotype of *bri1-116;scr-4* and *bri1-116;wox5-1* double mutants unveil a role for *BRI1* signalling upstream of *SCR* and *WOX5* in maintaining the identity and organization of the QC cells. Similarly, the lack of obvious responses of the *PLT* reporters to BL and the phenotype of the *bri1-116;plt1;plt2* triple mutants suggest that BRs regulate stem cell activities partly independently of the *PLT* family members, although subtle effects could be masked by the dramatic phenotype of the triple mutant combination.

Although QC cells divide rarely (Dolan et al., 1993; van den Berg et al., 1995), increased BR levels might enhance the competence of QC cells to divide. In support to this hypothesis, the overexpression of *CYCD3;1*, which stimulates supernumerary divisions in the columella cells in a wild-type background (Riou-Khamlichi et al., 1999), rescues the defective columella cells numbers of *bri1-116* mutants. The effects of the *bes1-D* mutation and of BL treatments show that BRs promote stem cell differentiation. Thus, BRs seem to control the normal cell cycle progression of all meristematic cells, including the slowly dividing QC cells, preserving meristem size and overall root growth (Fig. 8).

Specific effects of BRs in the root stem cell niche

The root stem cell niche stands at the position of a maximum of auxin concentration and response (Sabatini et al., 1999; Friml et al., 2003; Grieneisen et al., 2007). Auxin orchestrates the gene regulatory networks that maintain the proliferative and totipotent states. The synergism between the effects of BRs and auxin has been long recognized (Nakamura et al., 2003; Nemhauser et al., 2004). The strength of the auxin maximum, as revealed by the expression of the reporter *DR5:GUS*, can be modulated by BR signalling: in *bri1-116* mutants, the area and intensity of the maximum are restricted, whereas they are enlarged by treatments with BL (Mouchel et al., 2006; Nemhauser et al., 2004; Nakamura

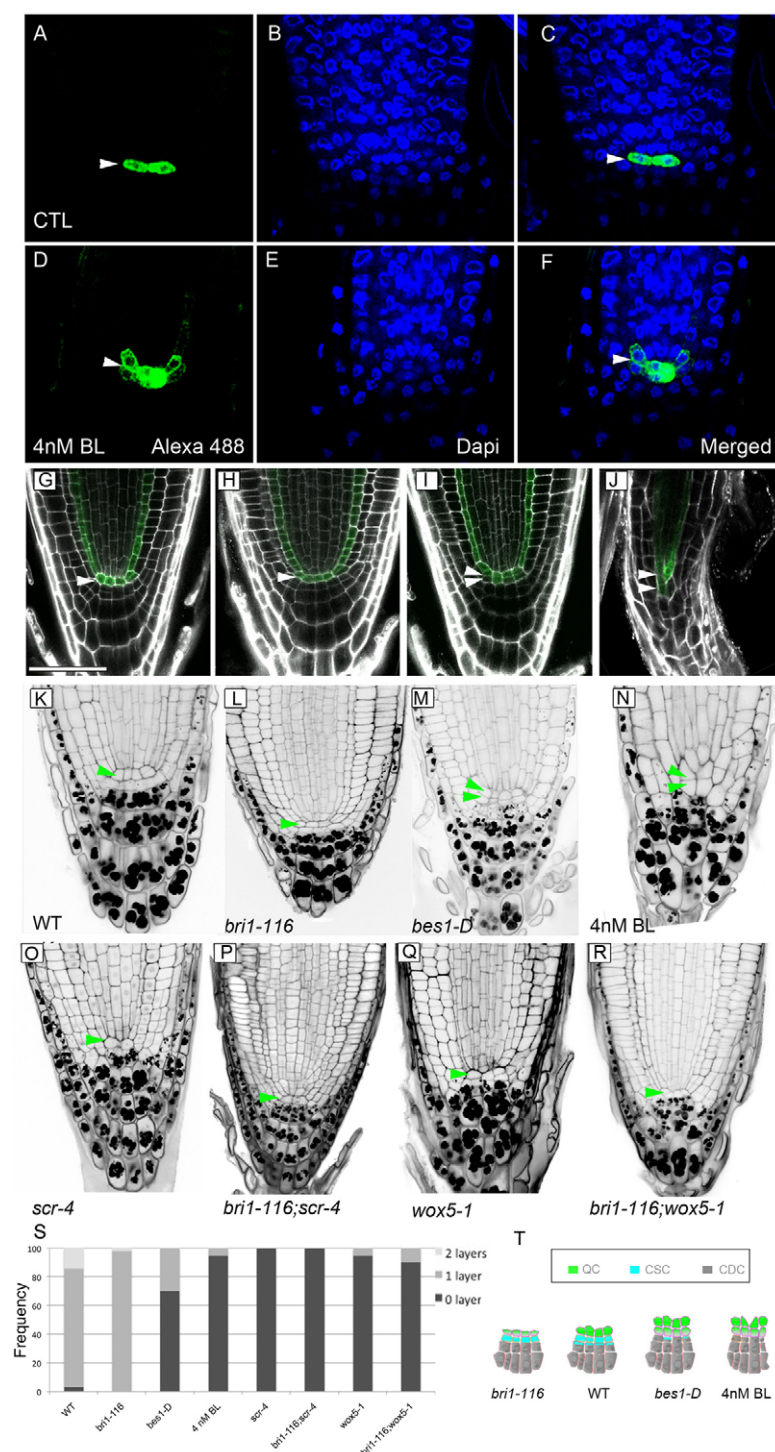


Fig. 7. Differentiation of distal columella stem cells promoted by BRs.

(A-F) Whole-mount immunofluorescence with anti-GFP antibodies used to label the QC cells of 6-day-old *pWOX5:GFP* plants (A-C) and *pWOX5:GFP* plants treated with 4 nM BL (D-F). Confocal images corresponding to Alexa488 fluorescence (A,D), DAPI staining (B,E) and combined DAPI+GFP (C,F). Arrowheads indicate the position of the QC cells in the root. (G-J) *pSCR:GFP* expression in the roots of Col-0 (G), *bri1-116* (H), *bes1-D* (I) and Col-0 plants treated with 4 nM BL (J). Scale bar: 50 μ m. Arrowheads indicate the position of the QC cells in the root. (K-N) mPS-PI-stained root tips of Col-0 (K), *bri1-116* (L), *bes1-D* (M) and Col-0 treated with 4 nM BL (N) (green arrowheads indicate cells with QC identity). (O-R) mPS-PI-stained root tips of *scr-4* (O), *bri1-116;scr-4* (P), *wox5-1* (Q) and *bri1-116;wox5-1* (R). (S) Quantitative analysis of the effects of BRs in CSC differentiation. Frequency distribution of the number of cell layers is given between the QC and the first differentiated columella cells that contain starch granules. (T) CSC differentiation phenotypes in roots with altered BR signalling. QC, quiescent centre; CSC, columella stem cells; CDC, columella differentiated cells.

et al., 2003). In this context, our results might reflect alterations of the auxin maximum in BR-related mutants. Auxin has recently been shown to repress the expression of *WOX5*, leading to a faster CSC differentiation (Ding and Friml, 2010). In this scenario, the weak auxin maximum in *bri1-116* mutants should stimulate the expression of *WOX5* and delay CSC differentiation and, conversely, BR-enhanced signalling should increase the auxin maximum, restrict *WOX5* expression and accelerate differentiation. As none of these features were observed, BRs have to play a role on their own in the stem cell niche homeostasis beyond their interactions with auxin.

Recently, a role for gibberellins in the control of cell proliferation has been demonstrated. In mutants unable to produce this hormone, meristem and overall organ sizes are reduced. This effect correlates with both an increased expression of cell-cycle inhibitors and a reduced expression of the *pCYCB1;1:GUS* reporter. Similar to our observation for BRs, the cell-cycle restraint caused by the absence of gibberellins can be overcome by the overexpression of *CYCD3;1* (Achard et al., 2009). However, neither gibberellin application nor depletion affect the expression of *WOX5*, *SHR* or *SCR*, further indicating that BRs play a specific role in the stem cell niche. The notion of the cell autonomous

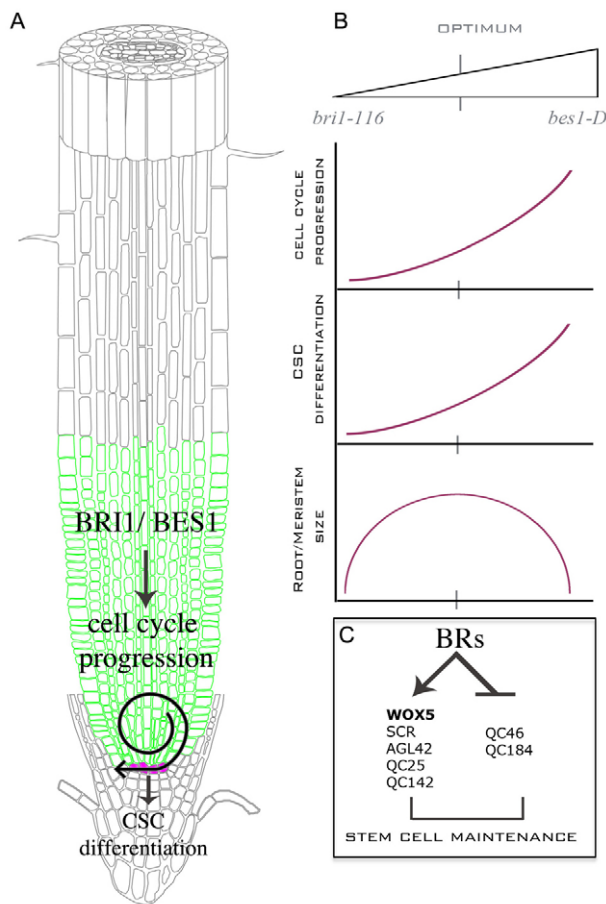


Fig. 8. Model for BR action during root meristem growth.

(A) Normal cell-cycle progression at the root apex controlled by the BR signalling pathway that maintains a balance between self-renewal of stem cells and differentiation and elongation rates of their daughter cells. (B) Graphical representation summarizing the dose-dependent effects of BRs in root development at three levels: cell cycle progression (top), CSC differentiation (middle) and root/meristem size (bottom). Reduced BRI1 signalling (as in *bri1-116* mutants) decreases both cell elongation and entry into the M phase, leading to the production of shorter meristems and, hence, a partial loss in the renewal activity of distal stem cells and a reduced supply of undifferentiated CSCs. The combination of a premature cell cycle exit with accelerated cell differentiation in *bes1-D* and BL-treated plants are also detrimental to meristem size and root growth. (C) At the stem cell niche, BRs play exclusive roles in QC renewal and stem cell differentiation upstream of known regulators of stem cell dynamics such as WOX5.

action of BRs (Savaldi-Goldstein et al., 2007) in the shoots of *Arabidopsis* has been extended to roots and the epidermis has been shown to be the tissue where BR signals (of as yet unknown nature) are produced to coordinate the growth of whole organs (Hacham et al., 2011). Moreover, the fact that a gain-of-function mutation in *BES1* recapitulates the effects of BL treatments suggests that BRs act downstream of this transcriptional regulator and not at a crossroad of the pathway between the membrane receptor and the signal output. The identification of BL-dependent transcriptional programs in specific root cell types (Birnbaum et al., 2003; Brady et al., 2007) will help understand the role of steroid hormones in meristem function in plants.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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