Brassinosteroid perception in the epidermis controls root meristem size

Yael Hacham1, Neta Holland1, Cristina Butterfield2, Susana Ubeda-Tomas3, Malcolm J. Bennett3, Joanne Chory2,4 and Sigal Savaldi-Goldstein1,2,*

SUMMARY
Multiple small molecule hormones contribute to growth promotion or restriction in plants. Brassinosteroids (BRs), acting specifically in the epidermis, can both drive and restrict shoot growth. However, our knowledge of how BRs affect meristem size is scant. Here, we study the root meristem and show that BRs are required to maintain normal cell cycle activity and cell expansion. These two processes ensure the coherent gradient of cell progression, from the apical to the basal meristem. In addition, BR activity in the meristem is not accompanied by changes in the expression level of the auxin efflux carriers PIN1, PIN3 and PIN7, which are known to control the rate of mitotic activity and differentiation. We further demonstrate that BR signaling in the root epidermis and not in the inner endodermis, quiescent center (QC) cells or stele cell files is sufficient to control root meristem size. Interestingly, expression of the QC and the stele-enriched MADS-BOX gene AGL42 can be modulated by BRI1 activity solely in the epidermis. The signal from the epidermis is probably transmitted by a different component than BES1 and BZR1 transcription factors, as their direct targets, such as DWF4 and BRox2, are regulated in the same cells that express BRI1. Taken together, our study provides novel insights into the role of BRs in controlling meristem size.

KEY WORDS: Arabidopsis, Brassinosteroids, Growth, Cell proliferation, Cell-cell communication

INTRODUCTION
The control of final cell and organ size is a fundamental question in the biology of all multicellular organisms. Root length is determined by the number of proliferating cells and their mature final size (Beemster and Baskin, 1998). The root is characterized by consecutive developmental zones along its proximal-distal axis. These zones form a gradient of renewed cells that proliferate, elongate and differentiate (Fig. 1A). In the root meristem zone, cells undergo repeated rounds of cell division. Subsequently, the cells exit the meristematic zone to become part of the elongation/differentiation zone (EDZ) where cells cease dividing, undergo rapid cell expansion and differentiate. The root meristem zone can be further divided into the apical and basal meristem zones (Beemster et al., 2003; Ishikawa and Evans, 1995; Verbelen et al., 2006; Zhang et al., 2010). The apical meristem is characterized by a high rate of cell proliferation, where cells do not exhibit a significant gain in size. In the basal meristem, also referred to as the transition zone between the apical meristem and the elongation zone, cell proliferation rate slows or stops and cells become larger. The quiescent center (QC, organizing cells), together with their surrounding stem cells, define the stem cell niche.

Brassinosteroids (BRs) are essential for normal plant growth and development, and mutants that are unable to synthesize or perceive BRs are dwarfs. BRs are perceived upon direct binding to the extracellular domain of the cell surface receptor kinase BRI1 (He et al., 2000; Li and Chory, 1997). The signal is then transmitted from the plasma membrane to the nucleus, where dephosphorylation of the transcription factors, BES1 and BZR1, allows them to homo- or hetero-dimerize and bind DNA to regulate the expression of hundreds of genes (He et al., 2005; Kim et al., 2009; Yin et al., 2005). BES1 and BZR1 induce or repress the expression of their direct-target genes upon binding to two identified cis-elements, E-BOX and BRRE. The latter is found in many genes, including the BR-biosynthesis genes, which undergo rapid inhibition by BZR1 in response to BRI1 activation (He et al., 2005).

Several studies have attributed the growth defects of BR mutants primarily to impaired cell expansion (Clouse and Sasse, 1998; Perez-Perez et al., 2002; Savaldi-Goldstein et al., 2007; Szekeres et al., 1996), with a smaller effect on cell division (Mouchel et al., 2004; Mouchel et al., 2006; Nakamura et al., 2006; Nakaya et al., 2002; Reinhardt et al., 2007). However, our knowledge of how BRs regulate root growth and meristem size is scant and systematic analysis is lacking. Multiple phytohormones contribute to the regulation of root growth. Auxin gradients, which are set up by the action of PIN auxin efflux carriers, control the extent of mitotic activity and differentiation (Galinha et al., 2007; Grieneisen et al., 2007). Cytokinins promote cell differentiation by inducing the expression of SHY2, a negative regulator of the expression of several PIN genes (Dello Ioio et al., 2008). Gibberellins (GA) promote cell expansion and the rate of cell proliferation through downregulation of cell cycle inhibitors (Achard et al., 2009; Ubeda-Tomas et al., 2009; Ubeda-Tomas et al., 2008). Finally, ethylene inhibits cell elongation through interaction with auxin (Ruzicka et al., 2009; Stepanova et al., 2005; Swarup et al., 2007).

Recent works have shown that the activity of plant hormones from a subset of cells can control growth and development of the entire organ (Dello Ioio et al., 2007; Savaldi-Goldstein et al., 2007;
Swarup et al., 2001; Swarup et al., 2005; Swarup et al., 2007; Ubeda-Tomás et al., 2009; Ubeda-Tomás et al., 2008). For example, BR signaling in the epidermis was found to both drive and restrict shoot growth (Savaldi-Goldstein et al., 2007). However, how BRs control meristem size remains unsolved. To address this issue, Arabidopsis roots were chosen as they represent a simplified developmental system, owing to a large number of available cell marker lines and the well-described radial organization of cell files that are accessible to imaging (Fig. 1A) (Petricka and Benfey, 2008).

Here, we show that the small meristem size of bri1 roots is attributed to both an impaired cell cycle activity and cell expansion. These defects result in a failure of cells to progress normally from the apical to the basal meristem. We further demonstrate that the size of the root meristem is controlled by BR1 activity in the epidermis. Thus, when present in the epidermis, BR1 initiates a signal which regulates gene expression of the meristematic inner cell files, i.e. AGL42. This signal is transmitted by a different component from BES1 and BZR1, which regulate their direct target genes locally. Taken together, we demonstrate the unique role of BRs in the control of root meristem size and suggest that cell-cell communication from the epidermis to the inner-meristematic cells is involved.

**MATERIALS AND METHODS**

**Plant material, growth conditions and chemical treatments**

All Arabidopsis thaliana lines and bri1-116 are in the Columbia (Col-0) background. Transgenic lines harboring the following transgenes have been described previously: pPLTS-er GFP (Galinha et al., 2007), pWOX5-er GFP and pSCR-H2B-YFP, pAGL42-GFP (Navy et al., 2005); pPIN1-PIN1-GFP (Benkovka et al., 2003) and CycB1;1-GFP (Ubeda-Tomás et al., 2009). Seeds were sterilized using a bleach solution with 1% hydrochloric acid and plated on 0.5% Murashige-Skoog medium (0.5 MS) (Duchefa Biochemie) supplemented with 0.8% (wt/vol) plant agar (Duchefa Biochemie). Plates were stratified in the dark at 4°C for 3 days and then transferred to 22°C in cycles of 16 hours light (25 ± 50 μmol m⁻² s⁻¹)/8 hours dark for 5 to 7 days. For chemical treatments, the BR biosynthesis inhibitor, BRZ, and BL were placed on the roots for 3 and 16 hours. Meristem size was measured according to the cortical cell files, i.e. AGL42. This signal is transmitted by a different component from BES1 and BZR1, which regulate their direct target genes locally. Taken together, we demonstrate the unique role of BRs in the control of root meristem size and suggest that cell-cell communication from the epidermis to the inner-meristematic cells is involved.

**RESULTS**

**BRs control root length by promoting cell expansion and maintaining normal cell number in the root meristem**

Root length is a result of integrated cell proliferation and cell expansion rates. To determine to what extent these processes are controlled by BR activity, we first characterized the rate of root elongation in bri1 and wild-type seedlings. As shown in Fig. 1B,C, wild-type seedlings continuously accelerated their root growth rate between days 2 to 6 after germination, and this acceleration slowed down after day 6 (Fig. 1C). By contrast, the roots of bri1 elongated at a lower rate with a delayed small acceleration between days 5 and 6. As a result, at day 7, the root of bri1 is approximately one-third the length of wild type. Similar behavior was obtained in measuring the meristem size (see Fig. S1A in the supplementary material). We next calculated the relative rate of cell expansion along the root (relative elongation rate (RLER)) for wild-type and and Baskin, 2000). Two-tailed t-tests comparing transgenic lines with Col-0 were performed using Microsoft Excel software (P<0.05, see Tables S2-S5 in the supplementary material).

**Confocal microscopy**

Fluorescence signals were detected using LSM 510 META confocal laser-scanning microscope (Zeiss) with a 25X water immersion objective lens (NA. 0.8). Roots were imaged in water supplemented with propidium iodide (PI, 10 μg/ml). PI, GFP, CFP and YFP were excited with the 561, 488, 458 and 514 nm laser, respectively. The fluorescence emission was collected at 575 nm for PI, between 500 and 530 nm band-pass for GFP, between 469 and 522 nm band-pass for CFP, and between 522 and 576 nm band-pass for YFP.
**Epidermal BRI1 controls root growth**

The maximum elongation rate of cells in the root meristem is subdivided into two developmental zones that are determined according to the cortex cells. The apical meristem is characterized by high rate of cell division and extends from the quiescent center (QC) to the first notable larger cortical cell; basal meristem starts from the end of apical meristem and ends at the start of elongation/differentiation zone (EDZ), where cells exceed more than twice their size, stop dividing and, hence, commence to differentiate. Cell borders are marked by PI (red). st, stele; en, endodermis; c, cortex; ep, epidermis; lrc, lateral root cap.

**bril** (Beemster and Baskin, 2000; Ubeda-Tomas et al., 2008). The maximum elongation rate of cells in **bril** was lower than in wild type, and **bril** cells ceased elongating at 500 µM from the root tip (Fig. 1D). Indeed, mature cortex cells in **bril** mutants were approximately half the length of wild type (Fig. 1E).

To test whether cell proliferation is also affected in **bril**, we measured both the size of the meristem and the number of meristematic cells present in **bril** and wild type. The meristem length was reduced in **bril** (Fig. 2A, left-most panel). In addition, when compared with wild type, **bril** had 20% fewer meristematic cells (Fig. 2B, left-most panel). The results show that BRs impact on meristematic cell number in addition to promoting cell expansion.

**BRs are required for normal cell cycle activity**

We next asked whether the reduced cell number observed in the root meristem of **bril** is a result of impaired cell cycle progression. Therefore, we used the G2/M phase cell cycle marker CYCB1;1-GFP to monitor the occurrence of cell divisions (Colon-Carmona et al., 1999; Sena et al., 2009; Ubeda-Tomas et al., 2008). As the meristem size of **bril** is smaller than that of wild type (Fig. 2A,B, left-most panels), we normalized the number of CYCB1;1-GFP-expressing cells to the number of meristematic cells. This normalization represents the proportion of meristematic cells that are present at the G2-M phase of the cell cycle (Table 1) (Dello Ioio et al., 2007). In **bril** lines, or in wild-type lines treated with BRZ, the fraction of cells at the G2-M phase was reduced by ~40% (Fig. 3A,B,C; Table 1). In addition, we performed quantitative reverse-transcriptase (qRT-PCR) expression analysis of CYCB1;1 and the cytokinesis marker **KNOlle** (Lauber et al., 1997). In this case, in order to overcome the difference in root length between samples, we normalized their expression level to **RCH1** transcript (Casamitjana-Martinez et al., 2003) (Fig. 3E). **RCH1** served us as reference for meristem size as it is specifically expressed in

**Table 1. Cell-division marker in **bril** lines and after BRZ treatment**

<table>
<thead>
<tr>
<th>Cyclin-expressing cells (x)</th>
<th>Meristem cell number* (y)</th>
<th>Proportion of meristematic cells present at the G2-M phase† (x/y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>12.6±0.78</td>
<td>32.1±0.62</td>
</tr>
<tr>
<td><strong>bril</strong></td>
<td>5.1±0.67†</td>
<td>23.5±1.97†</td>
</tr>
<tr>
<td>mock</td>
<td>19.2±1.26</td>
<td>27.8±0.82</td>
</tr>
<tr>
<td>BRZ</td>
<td>10.7±0.78†</td>
<td>25±0.48†</td>
</tr>
</tbody>
</table>

*Root-meristem cell number is expressed as the number of cortical cells in the meristem.
†The proportion of meristematic cells present at the G2-M phase is calculated by dividing the number of cells expressing CYCB1;1-GFP with the root-meristem cell number (Dello Ioio et al., 2007).

Results are presented as means±s.e.m.
meristematic cells and is not modulated by BRs (see Fig. S2 in the supplementary material). The results show that in agreement with the reduced cell cycle activity, the expression of CYCB1;1 and KNOLLE were reduced in bri1 by 50% when compared with wild type (Fig. 3E). In addition, reduced immunofluorescence signal of KNOLLE in response to BRZ treatment is consistent with a decrease in transcript levels (Fig. 3D). We therefore conclude that BRs are required for normal cell cycle activity.

In addition to impaired cell cycle activity, stem cell niche malfunction could also affect cell number in the root meristem (Dello Ioio et al., 2007; Scheres, 2007). We therefore grew lines of Arabidopsis with stem cell niche patterning markers in the presence and absence of BRZ (see Fig. S3 in the supplementary material). Among six markers tested, the fluorescence signal corresponding to pPLT1-erCFP and pWOX5-GFP was slightly reduced by BRZ treatment, and pAGL42-GFP signal showed a dramatic reduction (see Fig. S3A,B,D in the supplementary material; Fig. 6A; see Fig. S9A in the supplementary material). WOX5 promotes columella stem cell (CSC) fate in the distal meristem (Sarkar et al., 2007). Indeed, its moderate modification by low BR level is in agreement with the reduction in the CSC frequency observed in bri1, as reported and discussed in the accompanying paper by González-Garcia et al. (González-Garcia et al., 2011).

**BRs are important to maintain gradual cell progression in the meristem**

We hypothesized that the reduced cell cycle activity in bri1 will be associated with slow cell progression along the meristematic zones. Therefore, we analyzed the effect of BRs in two consecutive zones in the meristem, the apical and the basal meristem, using well defined morphological criteria (Beemster et al., 2003; Ishikawa and Evans, 1995; Verbelen et al., 2006; Zhang et al., 2010) (Fig. 1A). Cells in the basal meristem are larger than cells in the apical meristem (Fig. 2C); they cease dividing or divide at a very slow rate and do not yet undergo fast elongation. Indeed, we did not observe CYCB1;1-GFP expression in the cortical cells of the basal meristem (n=74). These criteria were applied to both wild-type and bri1. bri1 had marginally fewer cells in the apical meristem and reduced meristem length compared with wild type, a difference that was further accentuated in the basal meristem (Fig. 2A,B, middle and right panels). Specifically, the length of the basal meristem relative to the apical meristem was reduced by 60% and it had 40% fewer cells than wild type. This is in contrast to a much smaller reduction in the apical meristem size of the mutant and its corresponding cell number (34% and 14%, respectively). Finally, we repeated our analysis in the strong BR biosynthesis mutant cpd and observed a similar cellular behavior; the cell number in the basal meristem was more affected than in wild type of its own mutant background, whereas no significant drop in cell number was observed in the apical meristem (see Fig. S1B in the supplementary material). The dramatic drop in cell number in the basal meristem relative to the apical meristem suggests that bri1 cells fail to progress normally to the basal meristem.

Failure of cells to progress along the meristem could be also explained by slow or impaired cell expansion. We therefore calculated the average cell size in the apical and basal meristem of bri1. As shown in Fig. 2C, BRs affected cell expansion in the

**Table 2. Summary of the genotypes harboring BRI1-GFP**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Col-0</th>
<th>bri1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBRI1</td>
<td>pBRI1-BRI1-GFPox</td>
<td>bri1;pGL2-BRI1-GFP</td>
</tr>
<tr>
<td>pGL2</td>
<td>bri1;pSCR-BRI1-GFP</td>
<td>bri1;pSHR-BRI1-GFP</td>
</tr>
<tr>
<td>pSCR</td>
<td>bri1;pSCR-BRI1-GFP</td>
<td>bri1;pSHR-BRI1-GFP</td>
</tr>
<tr>
<td>pSHR</td>
<td>bri1;pSHR-BRI1-GFP</td>
<td>bri1;pRCH1-BRI1-GFP</td>
</tr>
<tr>
<td>pRCH1</td>
<td>bri1;pRCH1-BRI1-GFP</td>
<td></td>
</tr>
</tbody>
</table>
meristem, and the difference in cell size between wild-type and bri1 cells was more severe as cells progressed along the distinct zones. These results indicate that cells fail to expand normally in the meristem. Taken together, BRs promote mitotic cell cycle activity and cell expansion, thus ensuring the normal cell progression along the meristematic zones.

**BR-mediated control of meristem size does not involve changes in the expression level of the stele-localized PIN1, PIN3 and PIN7**

We next asked whether BR activity in the root meristem is associated with changes in the expression level of PIN1, PIN3 and PIN7, which mediate the balance between cell proliferation and differentiation (Dello Ioio et al., 2008). To achieve this, we performed qRT-PCR expression analysis in BL and BRZ treated roots (Fig. 3F-H). We detected no change in the transcript level of these PINs in the absence of BR signal, similar to their unchanged protein level (PIN1 and PIN7 is shown, Fig. 3I,J). In addition, short and long BL treatment had no effect (Fig. 3F-H). Thus, BR-mediated control of root meristem size occurs through a different mechanism from that proposed by the auxin and cytokinin models for promoting cell proliferation and differentiation, respectively.

**Epidermal BRI1 activity promotes cell expansion and cell proliferation**

We have shown that BRs maintains normal cell progression in the meristem by promoting cell proliferation and proliferation (Figs 1-3). We therefore asked how these processes are affected by the spatial activity of BRI1. BRI1 is expressed in all cell layers of the root (see Fig. S4 in the supplementary material). To study the spatiotemporal control of BR signaling in the root, we directed the expression of BRI1 to specific cell files in bri1, using the promoters pGL2, pSCR and pSHR (Helariutta et al., 2000; Lin and Schiefelbein, 2001; Wysocka-Diller et al. 2000) (Table 2; Fig. 1A; Fig. 4A). These promoters drive expression in the epidermis, endodermis, and QC and stele cell files, respectively (Fig. 4A). Expression driven by pGL2 in the epidermis is confined to atrichoblast (epidermal non-hair-cell) and appears also in cells belonging to the LRC (Fig. 4A; see Fig. S5A in the supplementary material). To study the contribution of meristematic BRI1 activity per se to root length, we also expressed BRI1 under the RCH1 promoter. Because BRI1-GFP expression in the transgenic lines exceeds endogenous BRI1 levels, we also used pBRI1-BRI1-GFPox (Table 2). This line is known to cause ubiquitous BRI1 overexpression (Friedrichsen et al., 2000).

We initially compared the rate of root growth and meristem size of the different transgenic lines with wild type. The bri1;pRCH1-BRI1-GFP line exhibited similar growth rate and slightly smaller meristem when compared with wild type (Fig. 4C,D). Thus, BR activity in the meristem determines meristem size.

The root length and root growth rate of bri1;pSHR-BRI1-GFP and bri1;pSCR-BRI1-GFP lines was reduced compared with wild type (Fig. 4B,C). In accordance, their meristem size was smaller (Fig. 4D). By contrast, pBRI1-BRI1-GFPox and bri1;pGL2-BRI1-GFP had a similar growth rate to wild type (Fig. 4B,C). Strikingly, bri1;pGL2-BRI1-GFP lines had bigger meristem than wild type and pBRI1-BRI1-GFPox (Fig. 4D). Thus, BR perception in the epidermis is sufficient to control root length and meristem size. This regulation does not appear to involve the activity of the BRI1 homologs BRL1 and BRL3, as the changes in their transcript levels do not correlate with the observed phenotypes (see Fig. S6 in the supplementary material) (Cano-Delgado et al., 2004; Zhou et al., 2004).

It is intriguing that BRI1 overexpression in the outer cell file, as in bri1;pGL2-BRI1-GFP, resulted in bigger meristem relative to wild-type, whereas ubiquitous overexpression of BRI1, as in pBRI1-BRI1-GFPox, did not (Fig. 4D; Fig. 5). We reasoned that the meristem phenotype in pBRI1-BRI1-GFPox was a result of an above-optimal BR response. To test our hypothesis, we performed a sensitivity assay to BRZ (see Fig. S7 in the supplementary material). As shown, pBRI1-BRI1-GFPox line was more resistant to the inhibitory effect of BRZ on root elongation when compared with either wild-type or to bri1;pGL2-BRI1-GFPox. We next asked whether the short root of bri1;pSHR-BRI1-GFP and bri1;pSCR-BRI1-GFP is a result of above-optimal BR activity in the inner cell files that limits growth. To examine this possibility, we crossed bri1;pGL2-BRI1-GFP with these lines. The resultant crosses...
exhibited long roots, similar to bri1;pGL2-BRII1-GFP (see Fig. S8 in the supplementary material). Thus, BRI1 activity in the inner cell files does not restrict growth, and inhibition of root growth probably depends on high BR activity in multiple cell files.

To examine how spatial BRI1 activity affects cell progression in the meristem, we performed cellular analysis in the apical and basal meristem (Fig. 5). bri1;pSHR-BRII1-GFP and bri1;pSCR-BRII1-GFP seedlings had reduced size of both meristematic zones compared with wild type (Fig. 5A,B). In addition, the number of cells significantly dropped only in the basal meristem (Fig. 5C,D) and cell expansion in the two zones was impaired (Fig. 5E,F). By contrast, the size of the meristematic zones of bri1;pGL2-BRII1-GFP lines and their corresponding cell number exceeded wild type, but they had similar cell size (Fig. 5C-F). These results suggest that BRI1 activity in the epidermis is sufficient to promote cell expansion and proliferation.

In agreement with the effect of the transgenic lines on cell proliferation, the expression level of CYCB1;1 and KNOLLE relative to RCH1 expression was lower when BRI1 was expressed from the inner cell files, but similar to wild type when BRI1 was expressed from the epidermis (Fig. 5G). This is in agreement with CycB1;1-GFP expression in bri1;pGL2-BRII1-GFP and bri1;pHR-BRII1-GFP (Fig. 5H).

Taken together, the results shown in Figs 4 and 5 suggest that BR signaling in the root epidermis is sufficient to control the gradual cell progression in the meristem. In addition, the positive correlation between cell size and cell proliferation in the different lines further demonstrates that these processes are affected by BR activity.

**The QC- and stele-enriched AGL42 is one target for a BRI1-mediated signal that is initiated exclusively in the epidermis**

To provide molecular evidence for a signal from the epidermis to the inner-cell files, we searched for genes that are specifically expressed in the inner cells and are modulated by BR activity. One such candidate was the QC marker AGL42 (Nawy et al., 2005) (Fig. 6A; see Fig. S9A in the supplementary material). In agreement with the pAGL42-GFP reporter lines, AGL42 showed the expected tissue-specific expression pattern [QC (which is included in the endodermis data set)] and stele (Fig. 6A,B; see Fig. S9A in the supplementary material) (Birnbaum et al., 2003). AGL42 transcript is dramatically reduced in BRZ-treated seedlings and in bri1, but we detected no change in its expression level in response to short BL treatment and a moderate increase after longer BL treatment (Fig. 6C). Therefore, we examined its expression in the different lines. Strikingly, AGL42 had lower expression level in bri1;pSCR-BRII1-GFP and bri1;pHR-BRII1-GFP, similar to bri1. By contrast, bri1;pGL2-BRII1-GFP lines exhibited high AGL42 expression compared with wild type. Hence, direct BRI1 activity in the QC and stele, where AGL42 transcript is normally enriched, did not modulate its expression. Instead, the expression of AGL42 was restored by BRI1-mediated signal that originates in the epidermis. Thus, we provide molecular evidence that BRI1, specifically expressed in the epidermis, controls gene expression in the inner cell files.

**The known BR signaling pathway, from BRI1 to BES1 and BZR1, acts locally**

BES1 and BZR1 are transcription factors known to operate downstream in the BRI1 signaling pathway. To test whether the signal from the epidermis to the inner-cell files requires their activity, we examined the BRRE element-containing BRox2 (CYP85A2) gene. BRox2 is a BR-biosynthesis gene whose expression level is rapidly negatively modulated by BR treatment and whose basal expression level is higher in the stele when compared with the epidermis and LRC (Fig. 6D) (Birnbaum et al., 2003; He et al., 2005). In bri1;pHR-BRII1-GFP roots, BRox2 basal expression level (mock) was lower than wild type.

Hence, the BR pathway is highly active in the stele of bri1;pHR-BRII1-GFP lines (Fig. 6D, right panel). In agreement, BRox2 expression level decreased in response BL treatment, ultimately reaching lower transcript level than BL-treated wild type. Low BR response resulted in high BRox2 expression levels and bri1;pGL2-BRII1-GFP roots had high basal expression levels of BRox2. Furthermore, bri1;pGL2-BRII1-GFP roots were less sensitive to BL treatment when compared with wild type.

![Fig. 5. Epidermal BRI1 activity promotes cell proliferation and cell expansion in the root meristem.](image-url)
Thus, as opposed to AGL42, BRI1 expression in the epidermis does not appear to affect early BR target in the stele (Fig. 6D). As a control, BES1 is not seen to move from the epidermis to the inner cells in roots (see Fig. S9B in the supplementary material).

Next, we tested whether BES1 and BZR1 activity in the stele could regulate genes in the epidermis. WAG2 is a BRRE element-containing gene expressed in the epidermis (Fig. 6E, left panel). WAG2 is positively regulated by BRs. Indeed, the basal level of WAG2 was higher in bri1;pGL2-BRI1-GFP and lower in bri1;pSHR-BRI1-GFP roots when compared with wild type. After BL treatment, WAG2 expression in bri1;pGL2-BRI1-GFP and bri1;pSHR-BRI1-GFP reached higher and lower levels, respectively, when compared with wild type (Fig. 6E, right panel). Thus, BES1/BZR1 activity in the stele does not appear to regulate their early target in the epidermis.

To examine the expression of early BR-response genes further, we established a pDWF4-GUS reporter line. The DWF4 promoter contains the BRRE element and is a direct target of BES1/BZR1 (He et al., 2005). In wild type, the DWF4 promoter fragment drives low GUS expression in the epidermis and LRC (Fig. 6F). We therefore crossed the pDWF4-GUS reporter line to bri1 and the transgenic lines. As expected, the GUS signal was remarkably increased in bri1 when compared with wild type, but remained confined to the epidermis (Fig. 6G; see also Fig. S9 in the supplementary material). High level of GUS expression was also detected in bri1;pSCR-BRI1-GFP and bri1;pSHR-BRI1-GFP (Fig. 6I,J). As shown in Fig. 6K, the GUS signal was observed only in the epidermis and LRC, similar to its pattern in the bri1 background (see Fig. S9 in the supplementary material). Hence, BRI1 activity in the inner cell-layers does not regulate BES1/BZR1 targets in the epidermis, in agreement with the qRT-PCR data (Fig. 6E).

Taken together, we conclude that BES1 and BZR1 act locally, suggesting that the signal from the epidermis to the inner cells is transmitted by a different component.

**DISCUSSION**

This study provides an explanation for the short-root phenotype of bri1 and uncovers a distinct mode of meristem-size control when compared with other hormones. Specifically, our work demonstrates that BRs are necessary to maintain a coherent...
Fig. 7. Model for BR-mediated control of root meristem size. (A) Reaching a critical cell size is an essential requirement for cell cycle progression, and BRs positively affect both processes. In bri1, cells fail to expand normally and have impaired cell cycle activity. These defects probably impinge on their ability to enter the mitotic phase and delay their progression from the apical to the basal meristem. This is further reflected in the relative number of cells in the two zones (represented by the colored columns). Cells are represented by gray boxes and cytokinesis by dashed lines. (B) BR signaling in the epidermis is sufficient to control root meristem size, whereas BR signaling in the inner cell files (i.e. stele) is not. This indicates that the inner cells must receive instructive signal(s) initiated by BRI1 exclusively in the epidermis. The MADS-BOX gene AGL42 is a target of such a signal. The signal from the epidermis to the inner cells is transmitted by a different component from BES1 and BZR1, which regulate their direct target genes (i.e. BR-biosynthesis genes) locally in cells that express BRI1.

BRs promote cell expansion rate and mitotic cycle to maintain gradual cell progression in the meristem

We propose that BRs are required to drive specific stages of the cell cycle, probably before cell entry into the mitosis. Our assumption is based on the observation that the proportion of cells present at the G2-M phase and cytokinesis is lower in bri1 when compared with wild type (Fig. 3A-D). In addition, the number of cells in the apical meristem is less affected when compared with the severe reduction in cell number in the basal meristem. Hence, cell cycle progression is retained. Our assumption is also consistent with the ability of high CycD3;1 level to suppress the reduced number of cells in bri1, as reported in the accompanying paper by González-Garcia et al. (2011).

Does BR regulate the cell cycle machinery per se? Thus far, BRs have been reported to induce the expression level of two core cell cycle genes: CycD3;1 in suspension cells and CDKB1;1 in dark-grown seedlings (Hu et al., 2000; Yoshizumi et al., 1999). However, these genes have not been shown to be early targets of BRs. Moreover, in root meristematic cells, the transcript level of CycD3;1 is not affected by short and long BL treatment and by BRZ treatment (Y.H., unpublished). Likewise, CYCB1;1 and KNOLLE expression level is not responding to short BL application (Fig. 3E). Thus, whether BRs directly affect core cell cycle genes at the transcriptional or post-transcriptional level is currently an unanswered question.

It is well established that cell division and cell size are coordinated, and cell growth is an essential requirement for cell cycle progression (Jørgensen and Tyers, 2004; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). It is plausible that defects which determine how fast cells will double their size before division restrain cell cycle progression in bri1. Our data show that BRs are necessary to promote cell expansion rate and maintain normal cell size along the distinct root zonation (Fig. 1E; Fig. 2C). The size of cells in the meristem is a result of increasing cytoplasmic volume (Beemster et al., 2003). Although we do not exclude the involvement of BRs in the increase of cell-biomass, the role of BRs in regulating expansion processes through cell-wall modification is evident (Zurek et al., 1994). In addition, short BR treatment is known to positively regulate the expression of cell-wall organization enzymes required for cell expansion and division (Nemhauser et al., 2004).

BR activity represents an additional pathway for hormonal control of root meristem-size

Depending on their relative level, cytokinin and auxin promote cell differentiation and proliferation, respectively (Dello Ioio et al., 2008). Auxin gradients can prolong or shorten the distinct phases of proliferation and differentiation, and PIN proteins are essential for these processes (Bililou et al., 2005; Grieneisen et al., 2007). Cytokinin activity ultimately reduces the expression of PIN1, PIN3 and PIN7 in the stele to counteract auxin-positive regulation on cell division and initiate differentiation (Dello Ioio et al., 2008). Our data shows that BR activity is not associated with changes in the expression level of these stele-localized PINs (Fig. 3). Furthermore, cellular analysis indicates that in the presence of cytokinin, the root meristem has reduced cell number although the relative proportion of cells at the G2-M phase is not affected (Dello Ioio et al., 2007). The relative low proportion of cells at the G2-M phase in bri1 further argues against BR role in cytokinin/auxin interplay for determination of cell differentiation (Fig. 3). Positive interaction between BRs and auxin in promoting developmental gradient of cells in the meristem. This dynamics depends on BR-positive effect on cell cycle activity and cell-expansion rate. Remarkably, root meristem-size is controlled by BR1 activation in the epidermis, suggesting that the spatial regulation of both above- and below-ground organs may share common mechanistic principles. In the epidermis, BR1 activity induces signal(s) to communicate with the inner cells. This signal is transmitted by a different component than BES1 and BZR1, which act locally (Fig. 7).
root growth is evident through the action of BRX, which maintains optimal BR levels for auxin responses (Mouchel et al., 2006).

Remarkably, hormonal control of root meristem size can occur from a subset of cells. Hence, as opposed to cytokinin and GAs, which act in the transition zone of the stele and endodermis, respectively, we demonstrate that BRs are required in the epidermis (Dello Ioio et al., 2007; Ubeda-Tomas et al., 2009; Ubeda-Tomas et al., 2008).

The role of epidermal BRI1 activity in controlling meristem size

Our work clearly demonstrates that BRI1 activity in the epidermis promotes root meristem size. By contrast, BR-signal from the inner cell files (endodermis/QC and stele) had a lesser effect. Thus, BRs exert similar spatial regulation in both root and shoot (Savaldi-Goldstein and Chory, 2008; Savaldi-Goldstein et al., 2007). The simple organization of the root meristem combined with available cell type-specific gene expression data and reporter genes, allowed us to characterize the mode of BR activity and whether the inner cells in the meristem receive a signal from the epidermis.


