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RETINOBLASTOMA-RELATED Protein Stimulates Cell Differentiation in the *Arabidopsis* Root Meristem by Interacting with Cytokinin Signaling[W]

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Maintenance of mitotic cell clusters such as meristematic cells depends on their capacity to maintain the balance between cell division and cell differentiation necessary to control organ growth. In the *Arabidopsis thaliana* root meristem, the antagonistic interaction of two hormones, auxin and cytokinin, regulates this balance by positioning the transition zone, where mitotically active cells lose their capacity to divide and initiate their differentiation programs. In animals, a major regulator of both cell division and cell differentiation is the tumor suppressor protein RETINOBLASTOMA. Here, we show that similarly to its homolog in animal systems, the plant RETINOBLASTOMA-RELATED (RBR) protein regulates the differentiation of meristematic cells at the transition zone by allowing mRNA accumulation of *AUXIN RESPONSE FACTOR19* (*ARF19*), a transcription factor involved in cell differentiation. We show that both RBR and the cytokinin-dependent transcription factor ARABIDOPSIS RESPONSE REGULATOR12 are required to activate the transcription of *ARF19*, which is involved in promoting cell differentiation and thus root growth.

INTRODUCTION

In multicellular organisms, organ growth and development depends on tight coordination between cell division and cell differentiation. Plant postembryonic development takes place in localized growth regions called meristems. In the root meristem of the model plant *Arabidopsis thaliana*, stem cells organized in a stem cell niche generate transit-amplifying cells, which undergo additional divisions in the proximal meristem and differentiate in the meristem transition zone (TZ) (Benfey and Scheres, 2000; Dello Ioio et al., 2007). For meristem maintenance, and therefore continuous root growth, the rate of cell differentiation must equal the rate of generation of new cells: How this balance is achieved is a central question in plant development. We have previously shown that, in the *Arabidopsis* root meristem, the plant hormone cytokinin regulates the differentiation rate of transit-amplifying cells (Dello Ioio et al., 2007) by antagonizing the plant hormone auxin, which sustains cell proliferation (Dello Ioio et al., 2008). In particular, at the TZ, cytokinins are perceived by the ARABIDOPSIS HISTIDINE KINASE3 (AHK3) receptor (Higuchi et al., 2004; Nishimura et al., 2004; Dello Ioio et al., 2007), which initiates a phosphorelay signaling cascade leading to activation of two primary cytokinin response transcription factors, ARABIDOPSIS RESPONSE REGULATOR1 (ARR1) and ARR12 (Sakai et al., 2001; Dello Ioio et al., 2007). ARR1 and ARR12 activate the gene *SHORT HYPOCOTYL2* (*SHY2*) (Tian et al., 2002), a repressor of auxin signaling that negatively regulates the transcription of *PIN-FORMED* (*PIN*) genes (Blilou et al., 2005). Thus, cytokinin causes redistribution of auxin, affecting the position where cell differentiation is allowed (Dello Ioio et al., 2008). Conversely, auxin mediates degradation of the SHY2 protein (Tian et al., 2003), sustaining the activity of *PIN* genes and prompting cell division (Dello Ioio et al., 2008). This circuit is necessary to balance cell differentiation with cell division, thus regulating root meristem size and the overall rate of root growth.

In animals, a major regulator of both cell division and cell differentiation is the tumor suppressor protein RETINOBLASTOMA (pRB). pRB can form complexes with multiple partners, frequently through a conserved Leu-x-Cys-x-Glu (LxCxE) motif (Lee et al., 1998; Dick, 2007; Lendvai et al., 2007). In addition, pRB is known to promote cell cycle exit by binding to members of the E2F family of transcription factors, thus interfering with their ability to activate transcription of genes necessary for the G1-to-S transition (Yao et al., 2008; Henley and Dick, 2012).

An E2F-independent activity of pRB in cell cycle control has also been described (Alexander and Hinds, 2001; Ji et al., 2004; Binné et al., 2007) and relies on pRB binding to the S PHASE

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KINASE-ASSOCIATED PROTEIN2 (Ji et al., 2004) and to the ANAPHASE PROMOTING COMPLEX (Binné et al., 2007).

Besides acting as a corepressor of transcription, pRB can also operate as a transcriptional coactivator for cell type–specific transcription factors during differentiation (Korenjak and Brehm, 2005; Calo et al., 2010). For example, muscle differentiation depends on the ability of pRb to upregulate the transcriptional activity of the master differentiation inducer MyoD (Skapek et al., 2006), whereas bone differentiation depends on pRb coactivation of the RUNT-RELATED TRANSCRIPTION FACTOR2/CORE-BINDING FACTOR SUBUNIT ALPHA-1 protein (Lee et al., 2006; Luan et al., 2007).

The *Arabidopsis* pRB ortholog is the RETINOBLASTOMA-RELATED (RBR) protein (Gutzat et al., 2012). As in animals, RBR inhibits cell cycle progression by interacting with an E2F transcription factor homolog (Gutzat et al., 2012; Magyar et al., 2012). Recently, RBR has also been shown to directly interact with the SCARECROW transcription factor, regulating progression through a spatially confined asymmetric cell division in the *Arabidopsis* root stem cell niche (Cruz-Ramírez et al., 2012). In addition, local reduction of RBR in the root meristem expands the stem cell pool without altering cell cycle rates, suggesting that RBR levels generally regulate the differentiation of stem cell daughter cells (Wildwater et al., 2005). Increase of RBR levels in the shoot apical meristem results in stem cell differentiation (Wyrzykowska et al., 2006; Borghi et al., 2010). During the development of stomatal lineage, RBR has been shown to influence both the number of meristemoid mother cells (Borghi et al., 2010) and the last symmetric division of the guard mother cell (Weimer et al., 2012). These observations indicate that RBR has various roles in the regulation of cell differentiation and lead to the question of whether it also plays a role in the transition to overt differentiation in the root meristem.

Here, we show that RBR influences the differentiation of transit-amplifying cells at the *Arabidopsis* root meristem TZ by interacting with the AHK3/ARR12 cytokinin signaling module. In particular, we provide evidence that both ARR12 and RBR are required to activate the transcription of *AUXIN RESPONSE FACTOR19* (*ARF19*) (Okushima et al., 2005, 2007), which is also involved in promoting cell differentiation, thus modulating root meristem size and root growth.

RESULTS

RBR Influences Root Meristem Size

To investigate a possible role of RBR in the regulation of cell differentiation in the TZ, we used transgenic lines with reduced levels of RBR due to the expression of an artificial microRNA for gene-silencing overcome (amiGO) targeting the *RBR* sequence under the 35S promoter (*35S$_{pro}$:amiGORBR*) (Cruz-Ramírez et al., 2013). *35S$_{pro}$:amiGORBR* roots displayed an enlarged meristem with an increased number of meristematic cells that resulted in longer roots (Figures 1B, 1E, and 1F; see Supplemental Figure 1B online), suggesting that RBR acts as a negative regulator of root meristem size and root growth. To verify this suggestion, we analyzed the root phenotype of plants carrying a *35S$_{pro}$>>RBR* (*35S$_{pro}$:GAL4-VP16-GR*; *UAS:RBR*) construct, in which ectopic RBR activity can be induced by dexamethasone (dex) treatment (Wildwater et al., 2005). At 3 d postgermination (dpg), 12 h of dex induction were sufficient to reduce root meristem size and to inhibit root growth (Figures 1A and 1C; see Supplemental Figure 1A online), whereas at later stages of meristem development (5 dpg), longer induction times were needed to induce the same effects (Figure 1D). These data support the notion that RBR influences root meristem size and root growth and that it is more active in the initial growth phase of root meristem (at 3 dpg) than at later stages (5 dpg).

RBR Regulates Root Meristem Size without Interfering with Cell Division

We next asked whether the variation in root meristem size observed by manipulating RBR levels could be caused by the inhibitory activity of RBR on the cell cycle machinery or by the activation of an RBR-dependent cell differentiation program. We monitored cell cycle dynamics in the root meristem of *35S$_{pro}$>>RBR* plants, where RBR inducibility allowed us to distinguish between immediate and late responses.

We first followed the entrance of root meristematic cells into the S-phase using the 5-ethynyl-2′-deoxyuridine (EdU) nucleoside, an analog of thymidine, that is incorporated into DNA during active replication (Kotogány et al., 2010). We counted the EdU-stained nuclei in both wild-type and *35S$_{pro}$>>RBR* root meristems after 14 h of dex treatment and normalized their number to the number of meristematic cortex cells. Interestingly, we could not detect any significant difference between wild-type and *35S$_{pro}$>>RBR* plants, although the root meristem size of these plants was significantly reduced (Figures 2A and 2C).

We then crossed *35S$_{pro}$>>RBR* plants to plants harboring the *D-Box CYCB1;1:GUS* (for β-glucuronidase) construct (Colón-Carmona et al., 1999), which allows visualization of cells in the G2-M phase. After 14 h of RBR induction, the ratio between the number of GUS-stained cells and the number of meristematic cortex cells, as in the case of the EdU staining, was the same in untreated and dex-treated roots (Figures 2B and 2C).

These results suggest that the overall cell division rate in the proximal meristem is not primarily affected by RBR induction. This is in line with the observation that cell cycle duration in the root meristem is around 20 h (Campilho et al., 2006); thus, the rapid root meristem size decrease that we observed upon RBR induction (12 h) cannot be explained by a prominent action of RBR on cell division. We therefore concluded that RBR activity promotes premature activation of the differentiation program of root meristematic cells, thus leading to a decrease in root meristem size.

RBR Affects Root Meristem Size by Interacting with the *AHK3*/*ARR12* Pathway

The root phenotype of plants with a low level of RBR expression closely resembled that of the *ahk3-3*, *arr1-3*, and *arr12-1* cytokinin signaling mutants (Dello Ioio et al., 2007); accordingly, the root phenotype of plants with a high level of RBR expression resembled that of plants exposed to exogenous cytokinin (Dello Ioio et al., 2007). We therefore investigated the possibility that

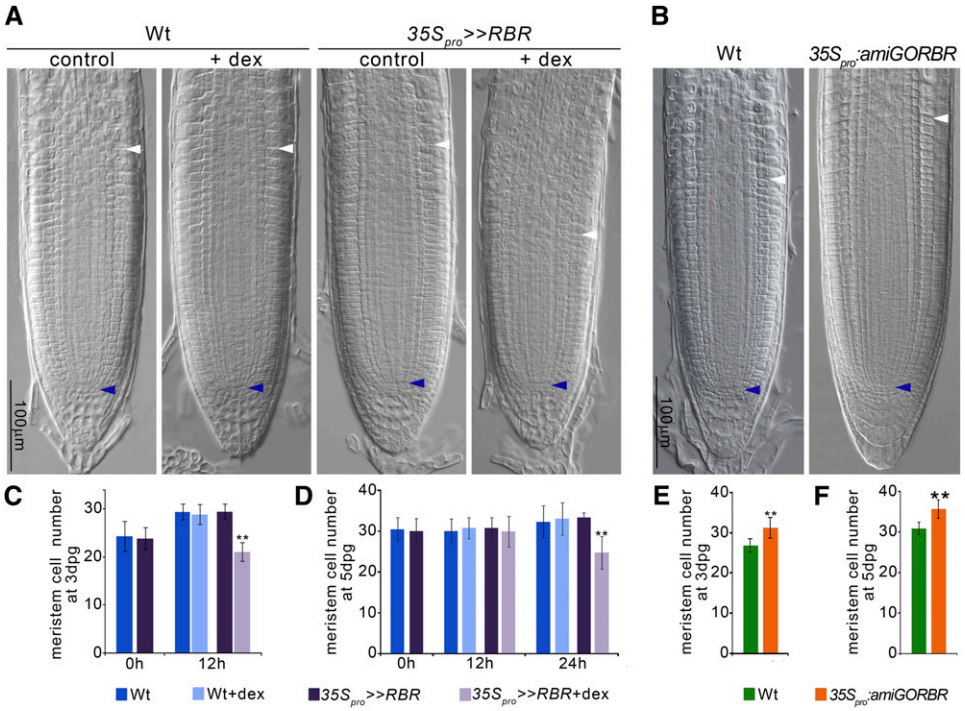

Figure 1. RBR Affects Root Meristem Size and Root Growth.

(A) DIC images of wild-type (Wt) and *35Spro>>RBR* root meristems treated with mock (control) or dex for 12 h. Blue arrowheads point to the quiescent center, and white arrowheads indicate the TZ.

(B) DIC images of wild-type and *35Spro*:*amiGORBR* root meristems, with blue arrowheads pointing to the quiescent center and white arrowheads indicating the TZ. Bars = 100 µm.

(C) Root meristem size, expressed as the number of cells in a cortex file from the quiescent center to the first elongated cortex cell (TZ) of 3-d-old wild-type and *35Spro>>RBR* plants before (0 h) and after 12 h of dex treatment.

(D) Root meristem size of 5-d-old wild-type and *35Spro>>RBR* plants before (0 h) and after 12 or 24 h of dex treatment.

(E) Root meristem size of 3-d-old wild-type and *35Spro*:*amiGORBR* plants.

(F) Root meristem size of 5-d-old wild-type and *35Spro*:*amiGORBR* plants. Error bars represent SD of results from three biological replicates. Asterisks indicate significant difference from the wild type (Student's *t* test; $P < 0.01$). Pictures are representative of the population analyzed in the experiment.

RBR affects differentiation of meristematic transit-amplifying cells by interacting with the AHK3/ARRs/SHY2 cytokinin signaling pathway.

We first compared the effects of RBR induction in the wild type and in the *ahk3-3*, *arr12-1*, *arr1-3*, and *shy2-31* (Dello Ioio et al., 2008) loss-of-function mutant backgrounds. Induction of RBR in either *arr1-3* or *shy2-31* mutants resulted in arrest of root growth and meristem consumption as observed in wild-type plants (Figure 3C; see Supplemental Figures 2A and 2C online). By contrast, both root meristem activity and root growth were totally unaffected in both *ahk3-3* and *arr12-1* mutant plants (Figures 3A to 3C; see Supplemental Figures 2A to 2C online).

These results suggest that RBR needs the activity of AHK3 and ARR12 to trigger cell differentiation and influence root meristem size. Notably, ARR12 is involved in regulating cell differentiation during the initial growth phase of the root meristem (Moubayidin et al., 2010), when RBR is more efficient in modulating root meristem size.

To understand the nature of the RBR–AHK3/ARR12 interaction, we first assessed whether RBR regulates AHK3 or ARR12 transcription and/or protein levels. Quantitative RT-PCR (qRT-PCR) experiments and analysis of *AHK3*:*GUS* and *ARR12*:*GUS* translational fusions revealed that neither AHK3 nor ARR12 mRNA and protein expression were affected in $35S_{pro}$*>>RBR* plants (see Supplemental Figures 2D to 2G online). Thus, RBR either interacts with the AHK3 and/or ARR12 proteins or regulates the activity of a factor downstream in the AHK3/ARR12 pathway. AHK3 and ARR12 proteins do not contain the LxCxE motif, suggesting that RBR and the AHK3/ARR12 module may regulate a common downstream factor.

ARF19 Acts Downstream of Cytokinin and Is Required by RBR to Regulate Root Meristem Size

When searching candidates for a possible AHK3/ARR12 downstream factor that RBR may interact with, we noticed that ARF19, a transcription factor involved in auxin signal transduction (Okushima et al., 2005, 2007), is expressed at high level at the TZ (Figure 4A) (Okushima et al., 2005; Rademacher et al., 2011). In addition, it was induced by cytokinin, as revealed by qRT-PCR

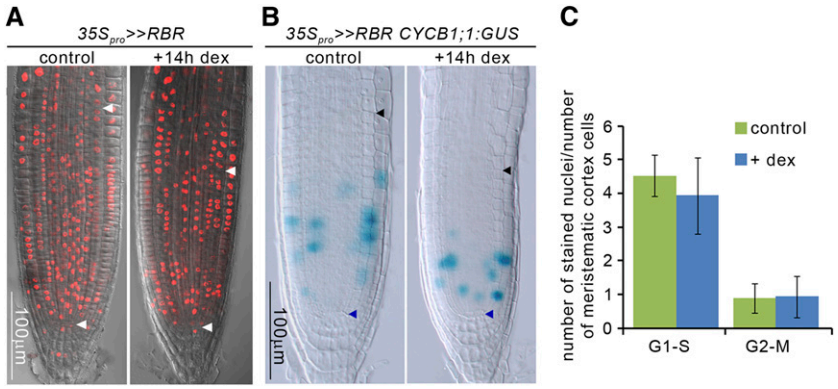

Figure 2. RBR Overexpression Does Not Interfere with Cell Division.

(A) EdU staining (red nuclei) of 3-d-old wild-type and *35Spro>>RBR* root meristems treated for 14 h with 2 µM dex. White arrowheads point to the quiescent center and the cortex TZ and indicate root meristem size. EdU-positive nuclei were visualized after 2 h of staining.
(B) *D Box CycB1:1 GUS* cell division marker in 3-d-old *35Spro>>RBR* roots treated with mock (control) or 2 µM dex for 14 h. Blue arrowheads indicate the quiescent center position; black arrowheads indicate the cortex TZ. GUS activity was visualized after 1 h of staining. Bars = 100 µm.
(C) Quantification of the experiments shown in **(A)** (G1-S) and **(B)** (G2-M). Columns represent the number of EdU-stained (left; G1-S) or GUS-stained (right; G2-M) nuclei normalized by the number of meristematic cortex cells (the region delimited by arrows in **[A]** and **[B]**). Error bars represent SD of results from three biological replicates. Pictures are representative of the population analyzed in the experiment.

analysis and exogenous cytokinin application to root meristems carrying a *ARF19*$_{pro}$:*GFP* (for green fluorescent protein) (Rademacher et al., 2011) transcriptional fusion (Figures 4A and 4B). To examine if ARF19 was the cytokinin-dependent factor regulated by RBR, we first assessed whether *ARF19* was affected by the AHK3/ARR12 regulatory module. As ARR12 activity depends on AHK3 function, the analysis was performed only in the *arr12-1* mutant background. *ARF19* promoter was less active in *arr12-1* roots compared with the wild type as visualized by the *ARF19*$_{pro}$:*GFP* promoter fusion (Figure 4A), and qRT-PCR experiments confirmed that the transcript also was reduced (Figure 4B). Induction of *ARF19* transcription by cytokinin was lost in the *arr12-1* mutant (Figures 4A and 4B), corroborating the idea that cytokinin regulates *ARF19* expression through the ARR12 transcription factor.

We next asked whether *ARF19* activity is important to determine root meristem size by analyzing the root meristem size of the *arf19-2* loss-of-function mutant (Okushima et al., 2005). Similarly to cytokinin signaling mutants and plants with lower RBR levels, *arf19-2* plants displayed larger root meristems and increased root growth compared with wild-type plants (Figure 4C; see Supplemental Figure 3 online).

These results indicate that *ARF19* transcription is regulated by ARR12 and that its activity is necessary to determine root meristem size. Thus, ARF19 could be the AHK3/ARR12 downstream factor targeted by RBR to regulate differentiation of transit-amplifying cells at the TZ.

As in the case of AHK3 and ARR12 proteins, ARF19 does not contain an LxCxE motif, so there are no indications that it can directly interact with RBR. We thus analyzed if RBR affects *ARF19* expression. Indeed, 3 h of RBR induction were sufficient to increase *ARF19* levels in *35S*$_{pro}$*>>RBR* roots, as visualized by both qRT-PCR (Figure 5B) and by analysis of *35S*$_{pro}$*>>RBR ARF19*$_{pro}$:*GFP* plants (Figure 5A). To confirm that ARF19 activity is required by RBR to reduce root meristem size, we analyzed the root phenotype of *arf19-2 35S*$_{pro}$*>>RBR* plants upon dex treatment. As for *ahk3-3 35S*$_{pro}$*>>RBR* and *arr12-1 35S*$_{pro}$*>>RBR* plants, the root meristem of *arf19-2 35S*$_{pro}$*>>RBR* plants did not decrease in size after RBR induction (Figure 5C; see Supplemental Figure 4 online). These results confirm that RBR acts through ARF19 to promote cell differentiation and to regulate root meristem size.

RBR Acts Together with ARR12 to Regulate *ARF19* Expression

We next asked whether RBR and ARR12 are both needed for the regulation of *ARF19* expression or they act independently. We first investigated the effect of RBR induction on *ARF19* transcription in the absence of ARR12. Interestingly, *ARF19* promoter activity in *arr12-1 35S*$_{pro}$*>>RBR* roots was lower compared with wild-type plants and could not be induced after RBR induction by dex treatment, as visualized by analysis of *ARF19*$_{pro}$:*GFP* in *arr12-1 35S*$_{pro}$*>>RBR* plants (Figure 5A); qRT-PCR confirmed that mRNA levels were lower in *arr12-1* (Figure 5B). This suggests that RBR needs ARR12 activity to promote *ARF19* transcription.

To assess whether RBR activity is required for the cytokinin-mediated activation of *ARF19*, we analyzed the effect of cytokinin (*trans*-Zeatin) on *ARF19* transcriptional activation in *35S*$_{pro}$:*amiGORBR* roots. The promoter activity of *ARF19* was lower in *35S*$_{pro}$:*amiGORBR* roots compared with the wild type as visualized by the *ARF19*$_{pro}$:*GUS* construct (Okushima et al., 2005) (Figure 5D), and qRT-PCR analysis revealed lower transcript levels (Figure 5E). Moreover, its transcription could not be induced by cytokinin treatment (Figures 5D and 5E). Thus, cytokinin-mediated activation of *ARF19* depends on RBR.

These data indicate that the cytokinin signaling pathway and RBR are required together to activate ARF19 expression.

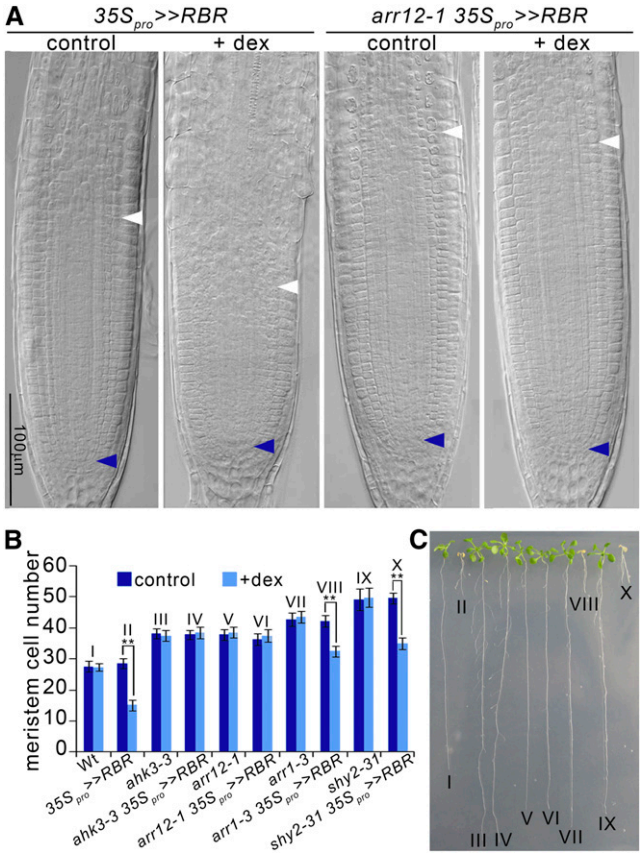

Figure 3. RBR Affects Root Meristem Size by Interacting with the AHK3/ARR12 Pathway.

(A) DIC images of root meristems, with blue arrowheads pointing to the quiescent center and white arrowheads indicating the TZ. RBR levels (*35Spro>>RBR*) were induced for 12 h by dex treatment in wild-type and *arr12-1* mutant backgrounds. Bar = 100 µm.

(B) Root meristem size, expressed as the number of cells in a cortex file from the quiescent center to the first elongated cortex cell (TZ) of 3-d-old plants treated with dex or mock (control) for 12 h. Roman numbers are the reference for **(C)**. Wt, the wild type.

(C) Picture of representative seedlings as in **(B)** after 7 d of dex treatment. I, wild-type; II, *35Spro>>RBR*; III, *ahk3-3*; IV, *ahk3-3 35Spro>>RBR*; V, *arr12-1*; VI, *arr12-1 35Spro>>RBR*; VII, *arr1-3*; VIII, *arr1-3 35Spro>>RBR*; IX, *shy2-31*; X, *shy2-31 35Spro>>RBR*.

Error bars represent SD of results from three biological replicates. Asterisks indicate significant difference from the wild type (Student's *t* test; $P < 0.01$). Pictures are representative of the population analyzed in the experiment.

DISCUSSION

Whereas animals and plants evolved distinct developmental strategies, growing evidence suggests that some key developmental mechanisms may be conserved between the two kingdoms (Nakagami et al., 2002; Beemster et al., 2003; Barrôco et al., 2005; Inzé, 2005; Wildwater et al., 2005; Cruz-Ramírez et al., 2012). In both kingdoms, the RETINOBLASTOMA protein has emerged as a key regulator not only of cell cycle withdrawal but also of cell differentiation. Here, we show that the plant counterpart, RBR, affects terminal differentiation in the *Arabidopsis* root meristem by regulating the position where transit-amplifying cells, generated by the stem cell niche, leave the meristem and enter the elongation/differentiation zone. We had previously shown that at this boundary (the TZ), cytokinin provides, via the AHK3/ARR/SHY2/PIN circuit, positional information instructing cells to stop dividing and to initiate their elongation/differentiation program (Dello Ioio et al., 2007, 2008; Moubayidin et al., 2010). We showed that ARR12 and ARR1 operate in the same districts of the root meristem, the TZ (Dello Ioio et al., 2007), and that they

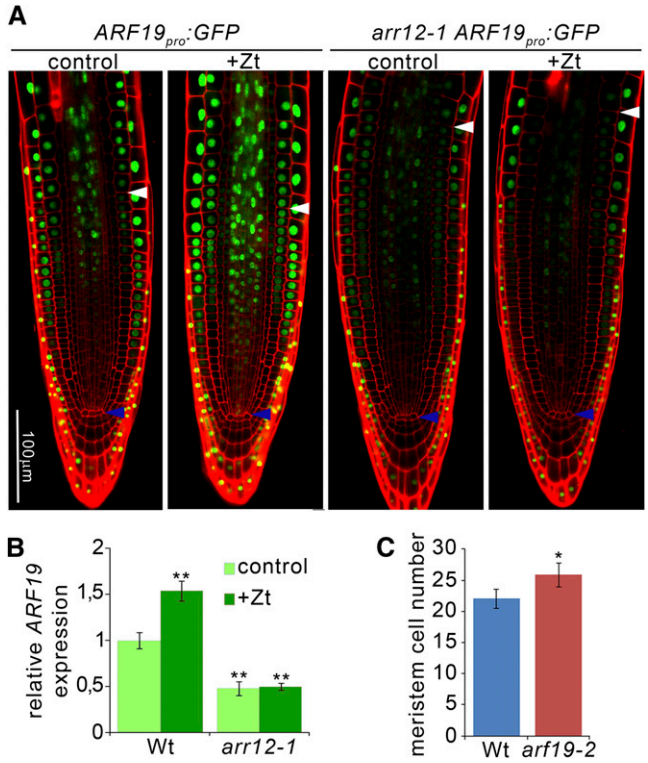

Figure 4. ARF19 Acts Downstream of ARR12 to Regulate Root Meristem Size.

(A) Confocal analysis of *ARF19pro*:*GFP* expression (green) in the root meristem of wild-type plants (Wt), the wild type treated for 4 h with 5 µM *trans*-Zeatin (Zt), *arr12-1* mutant plants, and *arr12-1* mutant plants treated for 4 h with 5 µM *trans*-Zeatin. The cell wall (red) was stained with propidium iodide. Bar = 100 µm.

(B) qRT-PCR of *ARF19* mRNA levels in the root tips of wild-type (Wt) and *arr12-1* mutant plants upon 4 h of 5 µM *trans*-Zeatin treatment. The value for the control mock-treated wild type was set to 1, and the relative values are shown.

(C) Root meristem size, expressed as the number of cells in a cortex file from the quiescent center to the first elongated cortex cell (TZ), of wild-type and *arf19-2* mutant plants at 3 dpg.

Experiments were performed on 3-d-old seedlings. Error bars represent SD of results from three biological replicates. Asterisk indicates significant difference from the wild type (Student's *t* test; $P < 0.01$ in **[B]**, and $P < 0.05$ in **[C]**). Pictures are representative of the population analyzed in the experiment.

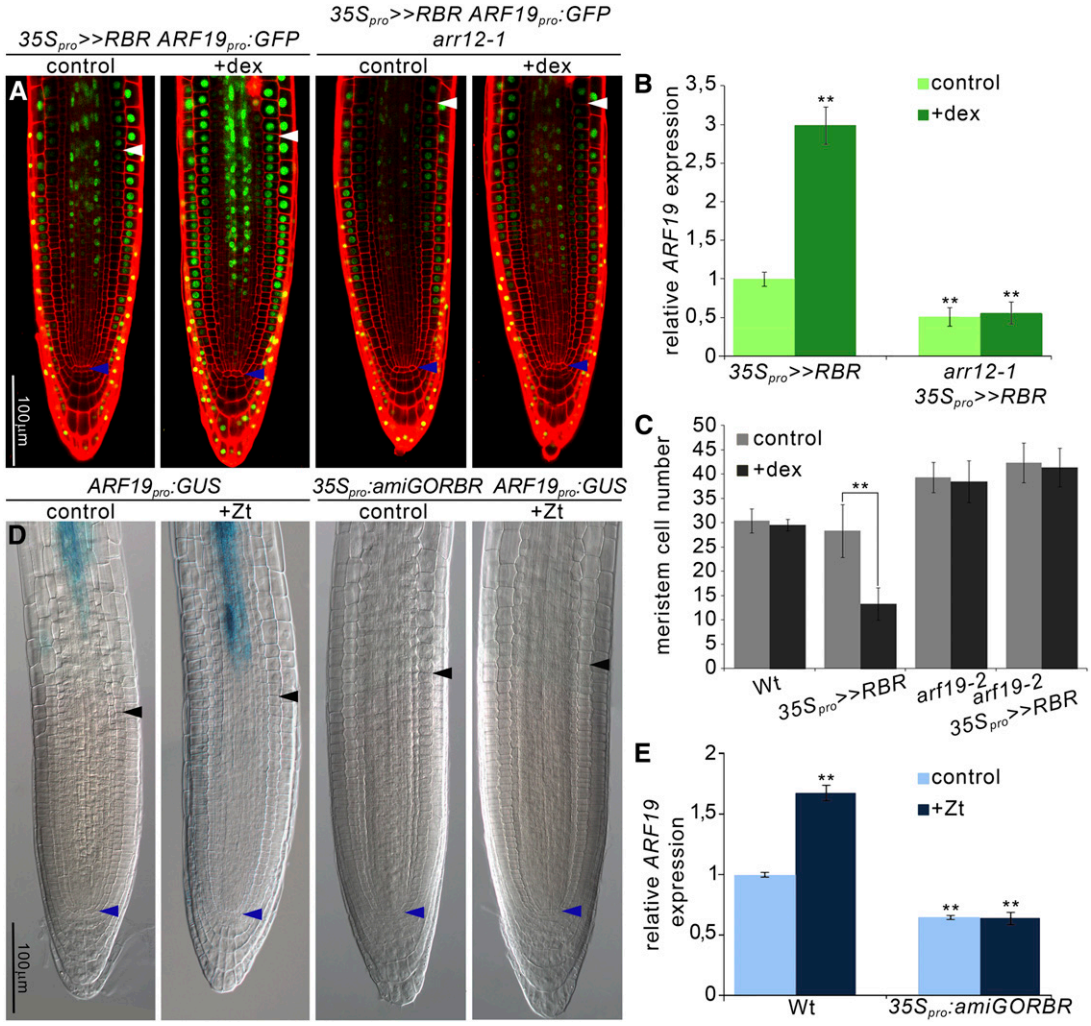

Figure 5. RBR and ARR12 Act Together to Regulate ARF19 Transcription.

(A) Confocal analysis of the variation in *ARF19pro*:*GFP* activity (green) in the root meristem of wild-type and *arr12-1* mutant backgrounds upon 3 h of RBR (*35Spro>>RBR*) induction by dex or mock (control) treatment. The cell wall (red) was stained with propidium iodide. Blue arrowheads point to the quiescent center, and white arrowheads indicate the TZ.

(B) qRT-PCR of *ARF19* mRNA levels in root tips of wild-type and *arr12-1* mutant plants upon 3 h of induction of RBR (*35Spro>>RBR*) by dex treatment. The value for mock-treated *35Spro>>RBR* (*35Spro>>RBR*; control) plants was set to 1, and the relative values are shown.

(C) Root meristem size at 3 dpg of wild-type (Wt) and *arf19-2* plants with RBR (*35Spro>>RBR*) induction by dex or mock (control) treatment. Plants that did not contain the *35Spro>>RBR* construct were also dex and mock treated as a control.

(D) DIC analysis of *ARF19* promoter activity in root meristems of wild-type and *35Spro*:*amiGORBR* plants expressing the *ARF19pro*:*GUS* construct. Plants were either mock treated (control) or treated with 5 µM *trans*-Zeatin (Zt) for 4 h. Blue arrows point to the quiescent center, and black arrows indicate the TZ. Bars = 100 µm.

(E) qRT-PCR of *ARF19* mRNA levels in root tips of wild-type and *35Spro*:*amiGORBR* plants treated with mock (control) or 5 µM *trans*-Zeatin for 4 h. The value for the control mock-treated wild type was set to 1, and the relative values are shown.

Experiments were performed on 3-d-old seedlings. Error bars represent SD of results from three biological replicates. Asterisk indicates significant difference from the wild type (Student's *t* test; $P < 0.01$). Pictures are representative of the population analyzed in the experiment.

act synergistically but at different stages of root meristem development (Moubayidin et al., 2010).

Our results indicate that RBR acts through the cytokinin signaling pathway, specifically via the AHK3/ARR12 branch, to activate *ARF19* transcription, thus regulating differentiation of transit-amplifying cells at the TZ and, hence, root meristem size (Figure 6).

Although RBR is ubiquitously expressed in the root meristem (Magyar et al., 2012), posttranslational modifications can make its function tightly constrained (Cruz-Ramírez et al., 2012). For example, in the root stem cell niche, RBR is specifically required in the ground tissue stem cell where an accurate spatiotemporal regulation of its phosphorylation status determines its ability to

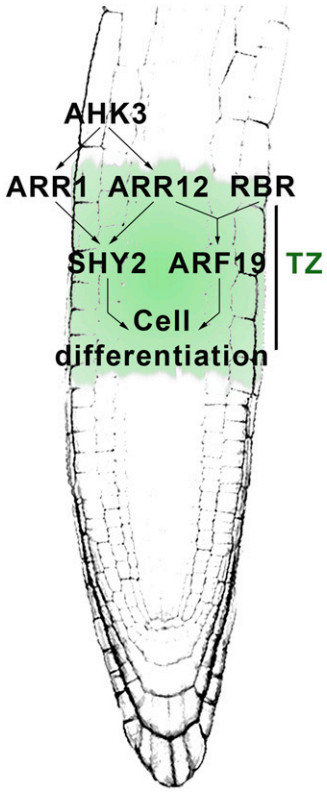

Figure 6. Model for the Cytokinin/RBR-Mediated Regulation of Cell Differentiation in the Root Meristem TZ.

Schematic view of a root meristem, with the domain of ARR12 expression highlighted in green. Cell differentiation at the TZ is ensured by the interconnected activity of two pathways. One is dependent only on cytokinin, where an AHK3/ARR1, ARR12 signaling module ultimately results in activation of *SHY2* (Dello Ioio et al., 2008). The other, which regulates *ARF19*, is dependent on both cytokinin, via ARR12 and RBR (this work).

bind and repress the SCARECROW transcription factor, thus regulating asymmetric cell division (Cruz-Ramírez et al., 2012).

ARF19 activation by RBR occurs mostly at the TZ: We therefore conclude that the ARR12/RBR pathway acts locally at this boundary to determine root meristem size. Indeed, ARR12 and the cytokinin signaling pathway in general act specifically at the TZ (Dello Ioio et al., 2007).

Moreover, the requirement of ARR12 but not of ARR1 for RBR activity suggests that RBR function is mainly required during the initial growth phase of root meristem development, when, as mentioned above, ARR12 plays a predominant role in regulating cell differentiation in response to cytokinin (Moubayidin et al., 2010). This is in line with the finding that RBR positively regulates the developmental switch from embryonic heterotrophic growth to autotrophic growth, acting as a repressor of late embryogenesis genes during germination (Gutzat et al., 2011). RBR activity is therefore pivotal during the first phases of meristem development.

To date, *ARF19* has been considered a positive regulator of auxin signaling, promoting cell division during auxin-mediated lateral root emergence (Wilmoth et al., 2005; Berckmans et al., 2011). However, primary root meristems of plants carrying *arf19-2* mutations are longer than the wild type (this work), and plants overexpressing ARF19 display shorter roots compared with the wild type (Okushima et al., 2005), suggesting that ARF19 acts as a negative regulator of primary root development, probably promoting cell differentiation.

In conclusion, cell differentiation at the TZ depends on, and is ensured by, the interconnected activity of two pathways: one, dependent only on cytokinin, impinging on the SHY2/PIN signaling module, and the other, dependent on both cytokinin and RBR, regulating ARF19 (Figure 6). The RBR-dependent pathway seems more active during the initial growth phase of root meristem development and does not require *SHY2*, as RBR induction in *shy2-31* mutants resulted in arrest of root growth and meristem consumption as observed in wild-type plants (Figure 3B; see Supplemental Figure 2A online). Nonetheless, both pathways contribute to TZ positioning and differentiation, ultimately determining root meristem size and root growth.

How RBR and ARR12 interact to affect *ARF19* expression is not yet clear. Although we have no support for a direct protein–protein interaction between RBR and ARR12, they may still be part of a large RBR-containing complex necessary to control transcription. Indeed, transcriptional activation of *ARF19* occurs only when both ARR12 and RBR are present. In *Arabidopsis*, RBR activity has been linked to its ability to act as a repressor protein, either by sequestering a specific protein via direct protein–protein interaction (Cruz-Ramírez et al., 2012; Magyar et al., 2012; Weimer et al., 2012) or by recruiting chromatin remodeling factors on the promoters of genes that are repressed via histone methylation and/or deacetylation (Ausín et al., 2004; Johnston et al., 2008; Jullien et al., 2008; Gutzat et al., 2011). Thus, RBR and ARR12 may activate *ARF19* expression by recruiting chromatin remodeling factor(s) on the promoter of an ARF19-repressing protein. Alternatively, RBR may interact with an ARR12-dependent protein responsible for *ARF19* transcriptional activation.

It should be pointed out that in animals, during both cell cycle repression and tissue differentiation, pRB acts as a transcriptional regulator primarily via a mechanism that consists of binding to the main transcription factor involved in the particular developmental process, regulating its *trans*-activating activity. For example, when pRb is bound to E2F, the complex acts as a growth suppressor and prevents progression through the cell cycle (Yao et al., 2008; Henley and Dick, 2012). During myogenesis, a transcriptional synergism between pRB and the bHLH transcription factor MyoD has been reported (Gu et al., 1993; Novitch et al., 1996, 1999; Sellers et al., 1998). Whether this cooperation depends on the physical interaction of pRb with MyoD or is mediated by the recruitment of a larger complex is still controversial (Smialowski et al., 2005), probably because pRb activity is generally context dependent and relies on its posttranslational modification.

Likewise, it is tempting to speculate that in the root meristem RBR may increase the transcriptional activity of ARR12 by recruiting the latter in a larger complex to the *ARF19* promoter. Further work is needed to understand the molecular basis of this interaction, for instance, by comparing ARR12 downstream target genes in the root meristems with root-specific RBR-interacting proteins.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) were used. *ahk3-3*, *arr1-3*, *arr12-1*, and *arf19-2* mutants are in the Col-0 background (Okushima et al., 2005; Dello Ioio et al., 2007, 2008), and the *shy2-31* mutant is in the L*er* background (Knox et al., 2003). $35S_{pro}$>>*RBR* (Wildwater et al., 2005) and $35S_{pro}$:*amiGORBR* (Cruz-Ramírez et al., 2013) transgenic lines are in the Col-0 background. *AHK3*:*GUS*, *ARR12*:*GUS*, $ARF19_{pro}$:*GUS*, and $ARF19_{pro}$:*GFP* transgenic plants have been described previously (Okushima et al., 2005; Dello Ioio et al., 2007; Rademacher et al., 2011).

For tissue culture conditions, *Arabidopsis* (Col-0 and L*er*-0 ecotypes) seeds were surface sterilized, and seedlings were grown on one-half-strength Murashige and Skoog (MS) medium containing 0.8% agar at 22°C in long-day conditions (16-h-light/8-h-dark cycle) as previously described (Perilli and Sabatini, 2010). For dex (Sigma-Aldrich) treatments, plants were transferred with tweezers onto MS plates containing 2 μM dex for the time required. To cope with the different ecotype backgrounds, wild-type, *shy2-31*, $35S_{pro}$>>*RBR*, and *shy2-31* $35S_{pro}$>>*RBR* plants were all selected from the segregating progeny of the *shy2-31* (L*er*-0) × *35S>>RBR* (Col-0) cross.

Root Length and Meristem Size Analysis

Root meristem size was expressed as the number of cortex cells in a file extending from the quiescent center to the first elongated cortex cell, excluded, as described previously (Dello Ioio et al., 2007; Perilli and Sabatini, 2010).

For each experiment a minimum of 30 plants were analyzed. Student's *t* test was used for data validation (http://graphpad.com/quickcalcs/ttest2.cfm).

GUS Histochemical Assay

GUS activity of transgenic plants (n = 40) was visualized as described (Willemsen et al., 1998). Staining of *AHK3*:*GUS*, *ARR12*:*GUS*, and *ARF19pro*:*GUS* was performed as previously described (Okushima et al., 2005; Dello Ioio et al., 2008).

For G2-M analysis, $35S_{pro}$>>*RBR D-Box CYCB1*:*GUS* , and *D-Box CYCB1*:*GUS* plants at 3 dpg were transferred to one-half-strength MS plates containing 2 μM dex for 14 h. Plants were then transferred into X-gluc solution [100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, and 1 mg/mL X-gluc], vacuum treated for 10 min, and incubated for 1 h at 37°C in the dark for staining. For analysis of cell division rate, GUS activity was visualized after 1 h of staining, and for each sample, the number of GUS-stained cells (X) and meristematic cortex cells (Y) were determined (Perilli and Sabatini, 2010). The cell division rate has been calculated as $X/Y \pm \sqrt{(\partial X/X)^2 + (\partial Y/Y)^2}$, where X and Y are the mean, and ∂X and ∂Y are their standard deviations. A minimum of 30 plants from three independent experiments was analyzed for each treatment or genotype. Student's *t* test was used for data validation (http://graphpad.com/quickcalcs/ttest2.cfm).

EdU Staining

EdU staining was performed as described (Kotogány et al., 2010). $35S_{pro}$>>*RBR* and Col-0 plants at 3 dpg were transferred to half-strength MS plates containing 2 μM dex for 12 h and then transferred for 2 h to liquid half-strength MS with 5 μM EdU (Invitrogen) + 2 μM dex or an equal amount of DMSO. Plants were then fixed in 2% formaldehyde, 0.1% Triton, and 1× PBS buffer for 40 min at room temperature. The signal was developed following manufacturer instructions (Click-iT EdU Alexa Fluor 555 Imaging Kit, C10338). Confocal images were acquired using a Zeiss LSM 710. For quantification, a minimum of 30 plants from three independent experiments was analyzed for each treatment or genotype. The number of EdU-stained nuclei from the quiescent center shootwards up to the TZ was counted and then normalized by the number of meristematic cortex cells. Student's *t* test was used for data validation (http://graphpad.com/quickcalcs/ttest2.cfm).

Differential Interference Contrast and Confocal Microscopy

Differential interference contrast (DIC) with Nomarski technology microscopy (Zeiss Axio Imager A2) was used to count meristem cell number and GUS staining visualization. Plants were mounted in chloral hydrate solution (8:3:1 mixture of chloral hydrate:water:glycerol).

ARF19pro:*GFP* plants were analyzed by confocal laser scanning microscopy using a Zeiss LSM 780. The cell wall was stained with 10 μM propidium iodide in water.

RNA Isolation and qRT-PCR

Total RNA was isolated from root tissues of 3-d-old seedlings using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the Superscript III first-strand synthesis system (Invitrogen). Transcript levels were monitored by qRT-PCR using gene-specific oligonucleotide primers (see Supplemental Table 1 online). qRT-PCR reactions were performed with SYBR Green SensiMix (Quantace) using an 7300 Real-Time PCR system (Applied Biosystems), according to the manufacturer's instructions. Data were analyzed using the ΔΔCt (cycle threshold) method and normalized with the expression of the reference gene *ACTIN2*. For each analysis, three technical replicates of qRT-PCR were performed on two independent RNA batches. Results were comparable in all experiments. Student's *t* test was used for data validation (http://graphpad.com/quickcalcs/ttest2.cfm).

Accession Numbers

Arabidopsis Genome Initiative numbers from this article are as follows: ACTIN2 (AT3G18780), AHK3 (AT1G27320), ARF19 (AT1G19220), ARR1 (AT3G16857), ARR12 (AT2G25180), CYCB1;1 (AT4G37490), IAA3/SHY2 (AT1G04240), and RBR (AT3G12280).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. RBR Influences Root Meristem Size and Root Growth.

Supplemental Figure 2. RBR Regulates Root Meristem Size in a Pathway Dependent on AHK3/ARR12 and Independent of ARR1/SHY2.

Supplemental Figure 3. ARF19 Affects Root Meristem Size.

Supplemental Figure 4. RBR Regulates Root Meristem Size in an ARF19-Dependent Manner.

Supplemental Table 1. List of qRT-PCR Oligonucleotide Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

S.P. and J.M.P.-P. planned and performed experiments. R.D.M., C.L.P., S.D.-T., M.D.B., E.P., and L.M. performed experiments. A.C.-R. provided

35S$_{pro}$:amiGORBR plants. P.C. and B.S. discussed experiments and provided critical review of the article. S.S. conceived the research and wrote the article.

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