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The *RETINOBLASTOMA-RELATED* Gene Regulates Stem Cell Maintenance in *Arabidopsis* Roots

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SUMMARY

The maintenance of stem cells in defined locations is crucial for all multicellular organisms. Although intrinsic factors and signals for stem cell fate have been identified in several species, it has remained unclear how these connect to the ability to reenter the cell cycle that is one of the defining properties of stem cells. We show that local reduction of expression of the *RETINOBLASTOMA-RELATED* (*RBR*) gene in *Arabidopsis* roots increases the amount of stem cells without affecting cell cycle duration in mitotically active cells. Conversely, induced RBR overexpression dissipates stem cells prior to arresting other mitotic cells. Overexpression of D cyclins, KIP-related proteins, and E2F factors also affects root stem cell pool size, and genetic interactions suggest that these factors function in a canonical RBR pathway to regulate somatic stem cells. Expression analysis and genetic interactions position RBR-mediated regulation of the stem cell state downstream of the patterning gene *SCARECROW*.

INTRODUCTION

Stem cells of multicellular organisms maintain themselves and generate daughters that may initiate developmental programs as simple as the replacement of a single cell type or as complex as the formation of an entire organism. Plants form organs throughout their entire life span, which can extend over 1000 years. For that purpose, they maintain pluripotent somatic stem cells within the meristems, local pools of mitotically active cells (Weigel and Jurgens, 2002). In the *Arabidopsis* root meristem (RM), stem cells for all root cell types surround a small group of organizing cells, the quiescent center (QC; Scheres et al., 1994) and together they form a stem cell niche. Laser ablation studies have demonstrated that the QC maintains the stem cell status of immediately surrounding cells (van den Berg et al., 1997). The QC is specified by combinatorial action of two gene sets. *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) encode AP2-type putative transcription factors whose transcripts are restricted to the QC and stem cells in response to distribution of the phytohormone auxin (Aida et al., 2004; Blilou et al., 2005). *SCARECROW* (*SCR*) and *SHORTROOT* (*SHR*) encode members of the GRAS family of transcription factors required for aspects of radial patterning but also for QC identity (Di Laurenzio et al., 1996; Helariutta et al., 2000; Wysocka-Diller et al., 2000; Sabatini et al., 2003). Loss of *PLT1* and *PLT2* or loss of either *SHR* or *SCR* results in defective QC specification, differentiation of stem cells, and meristem termination.

While the patterning mechanisms that position plant stem cells are beginning to be understood, it is unknown how these maintain stem cell division and prevent differentiation. In mammalian cells, the decision to enter a new cell cycle is made during the G1 restriction point. One of the key regulators of this restriction point is the retinoblastoma protein (RB; reviewed in Weinberg [1995]) which exerts its antiproliferative activity, at least in part, by inhibiting cell cycle promoting E2F transcription factors. Upon release from RB-mediated inhibition, E2Fs activate their target genes and allow cell cycle progression. In addition to its role in cell cycle progression, RB activity has been linked to maintenance of the differentiated state (Lipinski and Jacks, 1999). Recently, it has been shown that conditional inactivation of RB and/or its close homologs in mice stimulate cell cycle reentry and increase epidermal and neural precursor cell number, which makes the RB pathway an attractive candidate for the regulation of stem cell characteristics (Ruiz et al., 2004; Sage et al., 2003;

Vanderluit et al., 2004). Many mammalian systems, however, lack definitive stem cell markers, and it is difficult to pinpoint effects to true stem cells.

Plants contain homologs of RB, E2F, CDKs, cyclin D, and KIP-related proteins (KRPs), which suggests that a canonical G1-S regulatory pathway could be active (Inze, 2005). In vitro analyses support this notion, as plant RBR interacts with CYCD proteins (Huntley et al., 1998) and can be phosphorylated by a CDK2a/CYCD3 complex (Nakagami et al., 1999). *Arabidopsis* contains a single RB homolog, RETINOBLASTOMA-RELATED (RBR), that is required already in the haploid phase of the life cycle prior to fertilization (Ebel et al., 2004).

Here, we address postembryonic functions of the *RBR* gene using conditional loss-of-function and gain-of-function approaches. We show that *RBR* is transcribed in all mitotically active cells but that the stem cell population in the *Arabidopsis* root is particularly sensitive to RBR manipulation. RBR regulates the size of the stem cell population downstream of the *SCR* patterning gene and in a canonical CYCD/RB/E2F pathway. This connects stem cell niche patterning and stem cell maintenance in plants and indicates overlap between plant and animal stem cell maintenance mechanisms.

RESULTS

Arabidopsis RETINOBLASTOMA-RELATED Transcript Is Cell Cycle Regulated and Can Be Locally Reduced by Region-Specific RNAi

We investigated the transcriptional regulation of *RBR* after fertilization to gain insight into its potential roles at later phases of the life cycle. In situ hybridization with a *RBR*-specific probe reveals a salt-and-pepper distribution in all embryos up to the heart stage (n = 27), which suggests cell cycle regulation (Figures 1A and 1B). At early torpedo stage, *RBR* transcripts are retained predominantly in actively dividing root and shoot meristem anlagen (Figure 1C, arrowheads). In the postembryonic root meristem, *RBR* mRNA is elevated in a patchy pattern mostly within pairs of small cells (92% of intensely staining cells; n = 75), indicating that *RBR* transcription is highest after cells have completed a cell division cycle (Figure 1F). In the shoot, *RBR* transcription is restricted to the shoot apical meristem (SAM) and young leaf primordia (Figure 1I). Together, our data suggest a role for plant RBR in actively dividing cells.

Since *RBR* knockouts are gametophytic lethal (Ebel et al., 2004), we used the root meristem-specific RCH1 promoter, active from early heart stage onward in the root primordium and in the postembryonic root meristem (Casamitjana-Martinez et al., 2003), to drive an *RBR* RNAi construct in the root cell-division zone. Two independent *RCH1::RBR* RNAi (''rRBr'') lines showed silencing as determined by quantitative PCR (data not shown). To determine whether silencing was region specific, we performed in situ hybridization on rRBr embryos and seedlings. In early embryonic development, when the *RCH1* promoter is not active, *RBR* transcript distribution in rRBr is similar to wild-type (wt; Figure 1D). At torpedo stage, *RBR* expression vanishes in root primordia of rRBr lines (Figure 1E). After germination, *RBR* transcript is low in rRBr root meristems (Figure 1H), but wild-type levels can be observed in rRBr shoot apices (Figure 1J). We concluded that a root-specific RNAi system can significantly reduce *RBR* mRNA levels in embryos and postembryonic roots. Antibodies against RBR protein detected a 110 kDa protein in wt roots that was strongly reduced in roots from rRBr lines (Figure 1K), indicating that region-specific RNAi significantly affects RBR protein levels.

Reduction of RBR Leads to Supernumerary Stem Cells

In *Arabidopsis* roots, the columella root cap consists of a single layer of stem cells immediately distal to the QC and several tiers of differentiated cells that elongate and contain starch granules (Figure 2A). In 4 days postgermination (dpg) rRBr seedlings, additional undifferentiated cells appear in the columella stem cell area (Figure 2B). These cells encompass more tiers and occupy a larger area than wt stem cells (Figures 2C and 2P). This indicates that columella cells differentiate at a more distal position in rRBr plants. The differentiated columella cells in rRBr plants contain starch granules but reach smaller sizes (Figures 2B and 2F). The total area occupied by columella root cap cells steadily increases (Figure 2P).

To verify whether the QC is still functional in rRBr roots and whether the excessive undifferentiated columella cells are stem cells, we ablated the QC, which leads to columella stem cell differentiation in wt roots (Figure 2D; van den Berg et al., 1997). Ablation of QC cells in rRBr roots induces rapid differentiation of all undifferentiated columella layers (Figure 2E). We concluded that the QC is functional in rRBr lines and that additional stem cell layers accumulate in the columella root cap.

Lateral root cap (LRC) stem cells, which also act as epidermal stem cells, perform a characteristic periclinal cell division that creates new layers (Figure 2G, white arrowheads). In rRBr plants, 1–2 additional layers of LRC tissue are present (Figure 2H), suggesting additional stem cell activity. Promoter fusions of the *GL2* and *WER* genes mark the epidermis, but they are less active in LRC/epidermal stem cells of the wt (Lee and Schiefelbein, 1999; Masucci et al., 1996). Excessive cells with lower activity of these promoters are present in rRBr plants (Figures 2I–2L), consistent with an increased domain of LRC/epidermal stem cell activity. Cell size of LRC cells becomes progressively reduced (Figures 2H, 2J, and 2L), but LRC identity marker N9099 remains expressed in fully mature cells (Figures 2M and 2N). Our data suggest that, in both columella and LRC/epidermis, excessive stem cells accumulate which eventually differentiate; mature root cap cells gradually reach smaller final sizes.

While the number of ground tissue stem cells and their cortex and endodermis daughter cell files in circumference is 8 in the wild-type (Dolan et al., 1993), rRBr plants contained between 10 and 14 ground tissue stem cells and derived cell files (Figure 2O; n = 13). A stem cell-specific probe for ground tissue stem cells expanded in a significant proportion of rRBr plants (Figures 3P and 3R; see below), indicating that

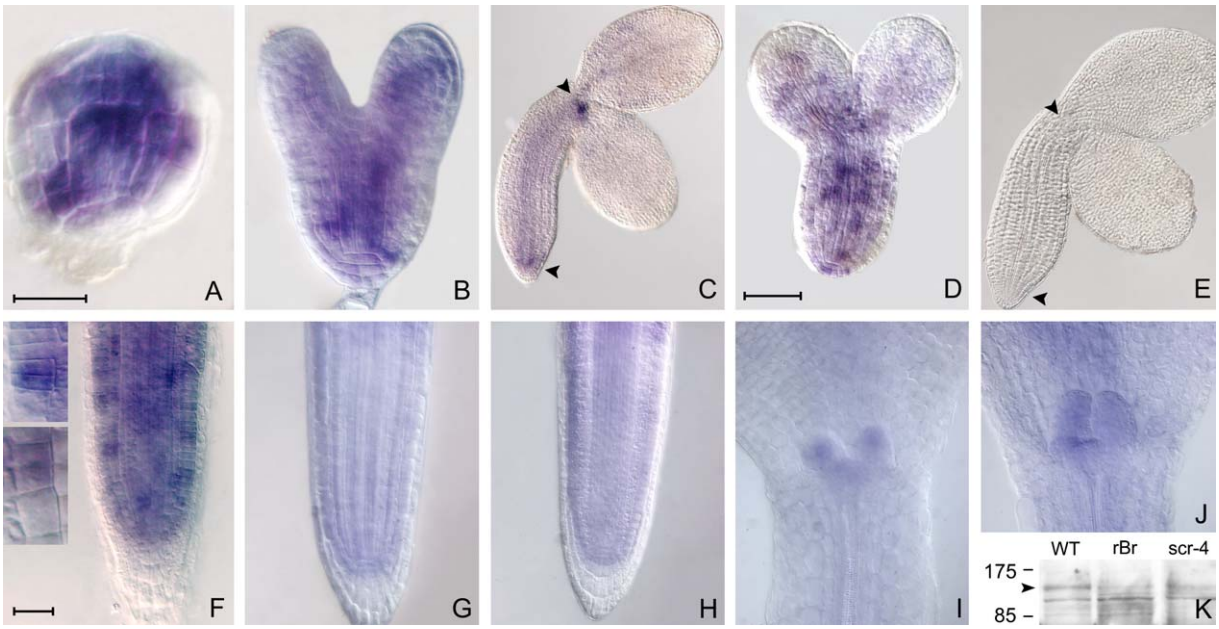

Figure 1. RBR Transcript Accumulation Is Downregulated in *RCH1::RBR* RNAi Lines

DIC images from whole-mount in situ hybridization signals (purple) in embryos and roots using an *RBR* specific probe.

(A–C) *RBR* transcripts in embryo development. Globular stage (A), heart stage (B), and torpedo stage (C).

(D and E) *RBR* mRNA in rRBr embryos. Expression is observed at late heart stage before the promoter driving *RBR* RNAi is active (D) but disappears at later stages (E).

(F–H) *RBR* transcripts in the root meristem. wt, insets shows pairs of cells with elevated signal (F); wt sense control (G); reduction of *RBR* transcript in rRBr root meristems (H).

(I and J) *RBR* transcripts in shoot apical meristem of wt (I) and rRBr (J) plants.

(K) RBR protein in wt, rRBr, and *scr-4* plants.

Bar, 50 μm in (A) and 25 μm in other panels.

ground tissue stem cell attributes expand in response to RBR reduction. Finally, provascular cell number was significantly increased at the stem cell level from 23–25 in wt to 32–37 in rRBr plants (Figure 2O; n = 9). Although consistent with increased stem cell activity, vascular stem cell-specific markers will be needed to substantiate this result.

In the proximal meristem, cells undergo several division rounds before they rapidly elongate and differentiate. rRBr root length, meristem size, meristem cell number, and size of differentiated epidermal and cortical cells are not significantly different from wt (Figures 2Q–2T), which indicates that the size of the proximal mitotic cell pool and postmitotic cell expansion are not affected in rRBr plants.

RBR Reduction Affects Stem Cell Fate Downstream of the SCR Patterning Cue

SCR, *SHR*, and the *PLT* genes are required for QC and stem cell patterning, and the expression of SCR and SHR protein fusions and *PLT1* promoter activity is not significantly altered in rRBr plants even at late developmental stages (Figures 3A–3C and 3F–3H). Also the quiescent center-specific markers QC25, QC46, and QC184, which depend on SHR, SCR, and PLT input, are initially correctly expressed, but switch off at later stages (Figures 3D, 3E, 3I, and 3J and data not shown). Together with our laser ablation studies which suggest that the QC in rRBr plants remains functional, these observations indicate that RBR does not influence stem cell fate through changes in cell patterning.

scr, *shr*, and *plt1,plt2* double mutants cannot maintain their meristems due to defects in QC specification and loss of stem cells. To find out if reduction of RBR activity maintains stem cells downstream of *SCR*, *SHR*, or *PLT* genes, rRBr was combined with strong mutant alleles for these genes. *scr-4*,rRBr plants retain undifferentiated columella and LRC cells (Figures 3L and 3N). QC ablation in this background causes rapid differentiation of these cells (Figure 3O), indicating that QC function is restored in *scr-4*,rRBr roots and that columella stem cells are present. At 14 dpg, when the meristem of *scr-4* mutants is completely consumed, *scr-4*,rRBr roots retain a large meristem. Additional cells files are created by excessive periclinal cell divisions in vasculature and ground tissue of *scr-4*,rRBr roots suggesting that, in the *scr-4* background, RBR reduction leads to excessive activity of all root stem cells (Figure 3N).

Large numbers of undifferentiated cells in *scr-4*,rRBr indicate that the transition to cell differentiation is compromised, a feature not seen in *scr-4* or outside the root cap area of rRBr plants. As these cells hardly expand, they barely contribute to root growth which is consequently not restored in *scr-4*,rRBr plants (Figure 2Q).

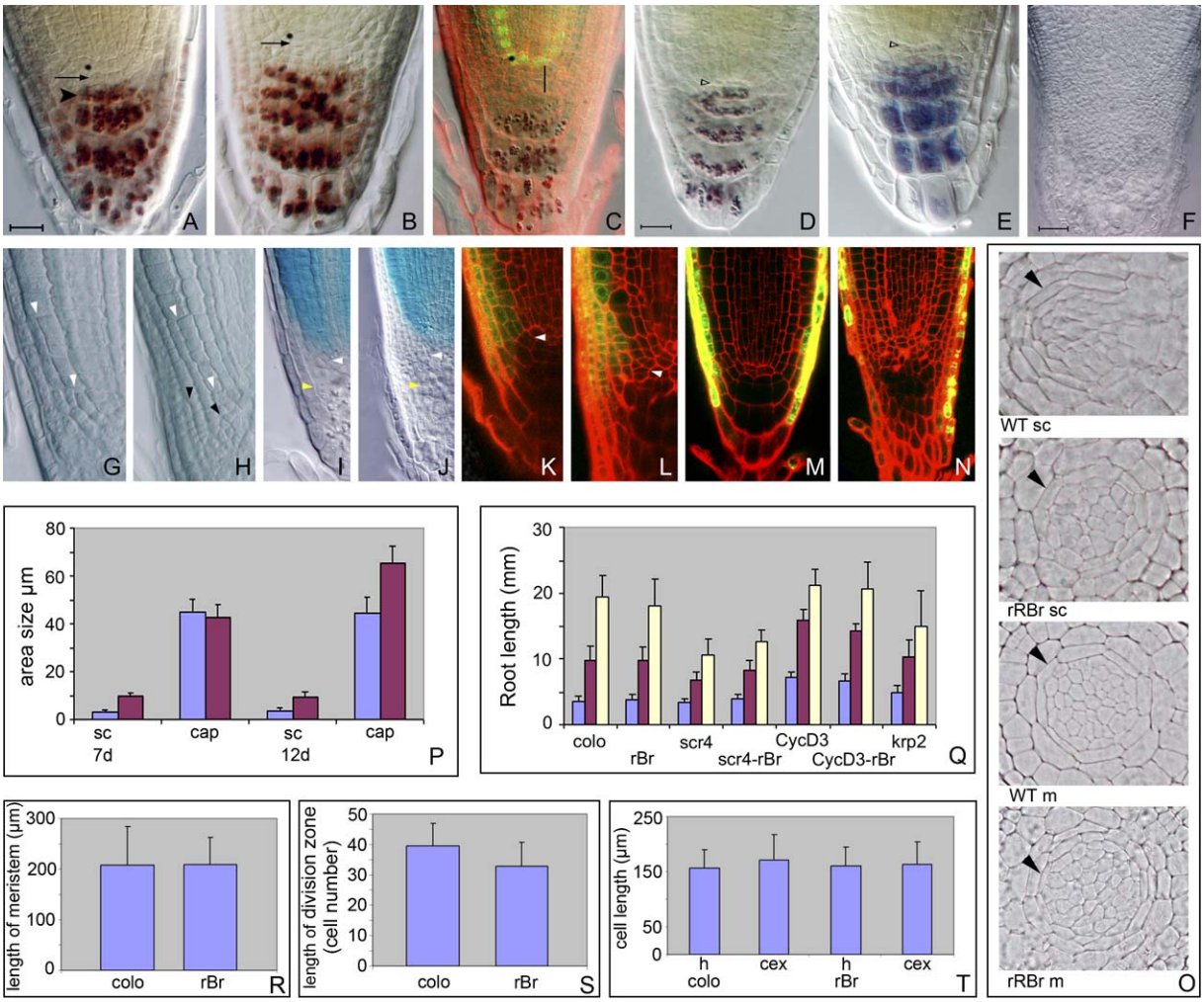

Figure 2. Reduction of RBR Leads to Supernumerary Stem Cells

(A–F) wt (A and D) and rRBr (B, C and E, F) root tip 4 (A–E) and 13 (F) days postgermination. No ablation (A, B, C, and F) and one day after QC ablation (D and E). Black arrowhead, starch granules in differentiated columella cells; arrow, columella stem cells; asterisk, QC; open arrowhead, ablated QC remnants.

(G and H) LRC in wt (G) and rRBr (H); white arrowheads, T divisions marking lateral root cap layers; black arrowheads, additional T divisions; yellow arrowheads, differentiated columella cells.

(I and J) pGL2::GUS in wt (I) and rRBr (J) roots.

(K and L) pWER::GFP in wt (K) and rRBr roots (L).

(M and N) N9099 marker in wt (M) and rRBr (N) roots.

(O) Stem cell and meristem file numbers in wt and rRBr. Sections taken in stem cell area (sc) and higher up in the meristem (m).

(P–T) Morphometric analysis comparing length in proximodistal direction of stem cell area (sc) and differentiated cap area in wt (blue) and rRBr (red) (P); total root length at 3 (blue), 5 (red), and 7(yellow) dpg (Q); meristem length from QC to elongation zone (R); number of cells in the cortical layer of the meristem zone (S); and size of differentiated epidermal hair and cortical cells (T). Error bars represent standard deviation.

DIC images after GUS stain (blue) or starch granule staining (purple) (A, B, D, E–J, and O); CLSM images with GFP signal (green) and propidium iodide counterstaining (red) (K–N); overlay of DIC and CLSM images from same root (C). Bar, 10 µm in (G) and 25 µm in other panels. Black arrowheads in (O), endodermal cell layer.

To further investigate whether RBR reduction can rescue stem cell activity downstream of SCR, we utilized a probe derived from the *At5g44160* gene that is specific for ground tissue stem cells in early-stage roots (Figure 3P). *At5g44160* mRNA accumulation in stem cells strongly depends on SCR activity (Figure 3U and Table 1), and its expression domain in the ground tissue is expanded in rRBr roots (Figures 3Q, 3R, and 3V), consistent with an expansion of the stem cell population after RBR reduction. Importantly, *scr-4,rRBr* plants significantly restore the expression of *At5g44160* in the ground tissue layer (Figure 3W and Table 1), supporting rRBr action downstream of SCR.

To investigate how RBR might be regulated by SCR, we determined transcript and protein levels in *scr-4* mutants.

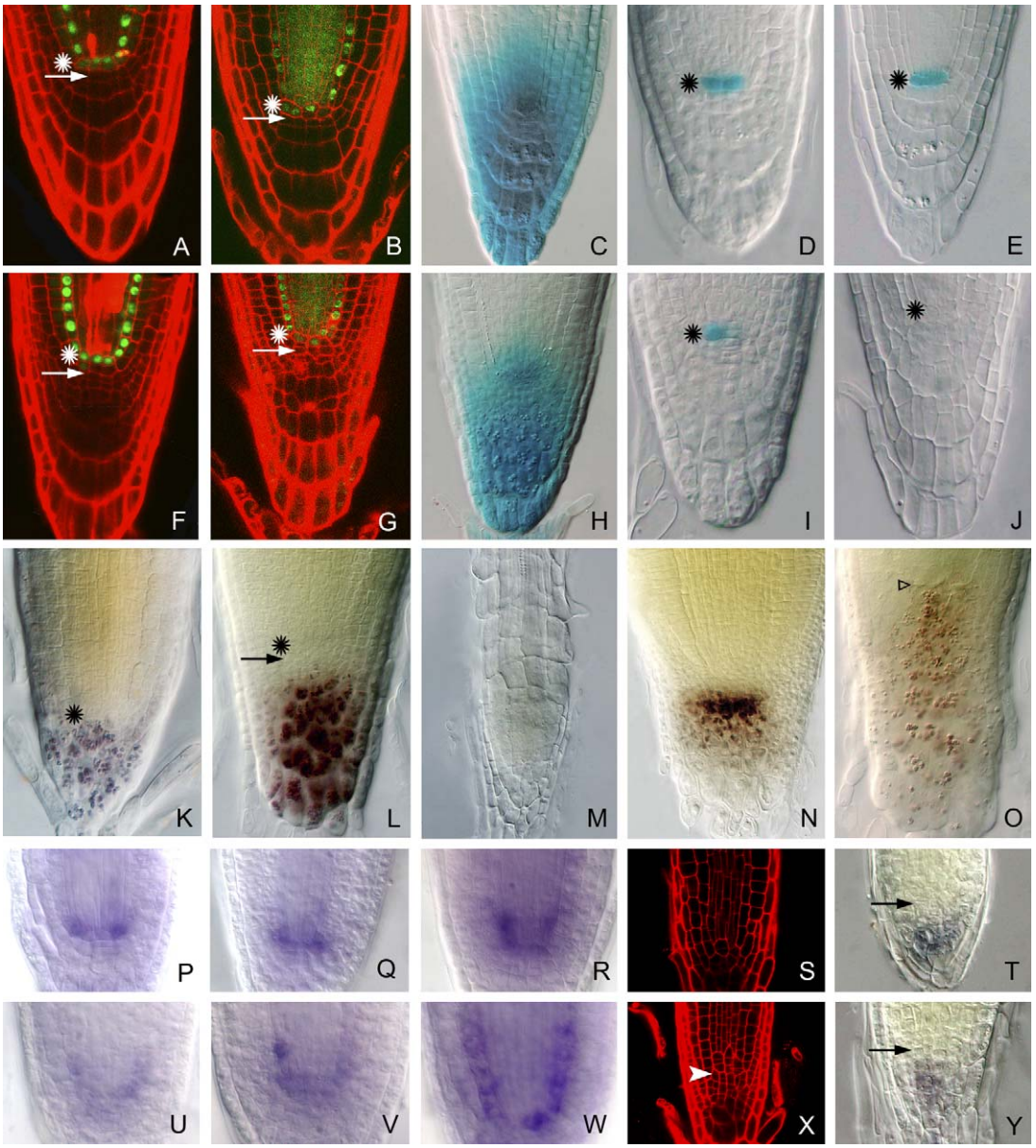

Figure 3. RBR Does Not Primarily Control Stem Cell Patterning Genes and Expression of QC-Specific Markers

(A–J) Patterning markers in wt and rRBr. wt (A–E) and rRBr roots (F–J) with SCR:GFP (A and F), SHR:GFP (B and G), pPLT-GUS (C and H) QC25 (D and I), and QC46 (E and J). Four days postgermination (A and F) and six days postgermination (B–E and G–J) roots.

(K–O) Genetic interactions between *RBR* and *SCR. scr-4* (K and M), *rRBr,scr-4* (L and N) rRBr,scr4 one day after QC ablation (O). Six days postgermination, (K) and (L); 7 dpg, (O); 14 dpg, (M) and (N).

(P–R and U–W) *At5g44160* mRNA as ground tissue stem cell marker in 3 dpg roots. wt (P), rRBr "normal" class (Q), rRBr "extended" class (R), rRBr "ectopic" class (V), residual expression observed in ~10% scr-4 roots (U), and rescue of expression in scr-4,rRBr (W).

CLSM images with GFP signal (green) and/or propidium iodide counterstaining (red) (A, B, F, G, S, and X); DIC images after GUS stain (blue), starch granule staining (purple) (C–E, H–J, K–O, T, and Y); DIC images with in situ hybridization signal (P–R and U–W). Arrow, columella stem cells; asterisk, QC; open arrowhead, ablated QC remnants; arrowhead in (X), cluster of small cells.

RBR transcript (data not shown) as well as protein (Figure 1K) was lower in *scr-4* mutants opposite to changes expected from SCR-mediated downregulation of RBR levels. Collectively, our data indicate that SCR acts upstream of RBR function and regulates both RBR activity and amount, but in opposite directions.

In the rRBr background, *shr-1* mutants contain a cluster of small undifferentiated cells in the stem cell area not present in

Table 1. Ground Tissue Stem Cell Marker Gene Expression

	SC	NSC	–	n
wt	111	0	32	143
scr-4	4[a]	5[a]	83	92
rRBr	59	21	78	158
scr-4, rRBr	2	16[b]	72	90

Quantification of *At5g44160* in situ hybridization data. Numbers of 3 dpg roots with staining focused in stem cells (SC), in non-stem cells (NSC), without staining (–), and totals analyzed (n).
[a] Weak staining in all roots.
[b] Weak staining in 25% of roots.

shr-1 alone (Figures 3S and 3X), but these completely differentiate after 5 dpg (n = 15), indicating only transient rescue of stem cell activity by RBR reduction in *shr-1*.

plt1,plt2,rRBr roots do not show any additional stem cell activity compared to *plt1,plt2* mutants at early stages when these still possess stem cells (Figures 3T and 3Y). Furthermore, *plt1,plt2,rRBr* roots differentiate at the same rate as *plt1,plt2* double mutant (n = 21). This full epistasis indicates that *PLT* gene function cannot be bypassed by RBR reduction.

RBR Reduction Maintains Stem Cell Properties in Daughter Cells

Additional cells in the columella stem cell area might arise as a consequence of an enhanced cell cycle in the single cell layer that performs these divisions in wt or by stem cell-like divisions in more layers. In the first scenario, the additional layers arise due to an inability of the differentiation process to keep up with the proliferation of daughter cells, whereas in the latter case the displaced daughters behave exactly like the stem cell layer. To distinguish between these possibilities, we used a novel in vivo time lapse recording procedure (B. Garcia et al., 2004, LNCS, abstract). We combined a plasmalemma marker LTI6b:GFP with the chromatin marker H2B:YFP to observe cell divisions in wt and rRBr roots. Unlike in wt, cell divisions can be observed in two layers of columella in rRBr roots (Figures 4A and 4B, arrows). The frequency of cell divisions in the rRBr stem cell region on a per-cell basis was not higher (Figure 4C). These data indicate that reduction of RBR maintains stem cell fate in columella daughters, rendering them competent to divide again.

In the meristem region proximal to the QC and stem cells, the number of divisions observed in time lapses is comparable between wt and rRBr plants (Figure 4C), supporting the notion that stem cells are more sensitive than the proximal meristem to changed levels in RBR. To analyze cell cycle progression in proximal meristem cells independently, we visualized cells in the G2-M phase using *D Box CYCB1;1:GUS* (Colon-Carmona et al., 1999). The total number of GUS-staining cells in wt and rRBr roots was similar (Figures 4D and 4E and data not shown), consistent with a similar overall division rate. *D Box CYCB1;1:GUS* was observed in the QC of ~30% of rRBr but not wt plants, suggesting that QC cells divide more frequently than in wt (Figure 4E, asterisk).

Acute Local Reduction of *RBR* Function Prolongs Stem Cell State and Affects Growth of Differentiating Root Cap Cells

The stem cell-specific defects and the reduction of cell expansion in rRBr plants could be due to variable levels of silencing of the *RBR* gene by the RNAi approach. To investigate whether a defined reduction in *RBR* gene copies could separate these effects, we utilized the gametophytic lethal *rbr1-3* allele. First, we noticed that *rbr1-3/RBR*$^+$ plants display subtle columella cell expansion defects and, more rarely, stem cell proliferation (see Figure S1 in the Supplemental Data available with this article online). Next, we sought to complement the gametophytic defect of *rbr1-3* by a single copy insertion of an 8.5 kb genomic *RBR* region between lox recombination sites (*pCB1-RBR*; Ebel et al., 2004; Heidstra et al., 2004). The genomic *RBR* fragment partially complemented gametophytic RBR requirement, which resulted in plants containing at least one wt *RBR* allele and additional *pCB1-RBR* alleles (see Experimental Procedures). Crosses to plants with heat-shock promoter-driven Cre recombinase (HS-CRE) allows heat-shock-induced removal of *RBR* copies between lox sites in clones marked by GFP (Heidstra et al., 2004).

pCB1-RBR and HS-CRE parental lines reveal no phenotypic defects after heat shock (data not shown). Clones in *rbr1-3/RBR*$^+$ background where either one or two copies of *pCB1-RBR* can be removed fall into three phenotypic classes. Plants with clones at both sides of the root cap show stem cell proliferation only (Figure 4F). Plants with clones that encompass part of the QC and root cap cells show stem cell proliferation and root cap bending (Figure 4G) or root cap bending only (Figure 4H). In the latter case, root cap bending occurs toward the region containing cells with excisions (n = 18). Interestingly, only clones associated with root cap bending can be found in *RBR*$^+$/*RBR*$^+$ background after removal of one copy of *pCB1-RBR* (data not shown). We concluded that a decrease from three to two RBR copies leads to a reduction in root cap cell growth (resulting in bending), which is consistent with the observed gradual reduction of columella cell size in RBR RNAi plants. Reduction from three to one copies leads to stem cell proliferation and attenuation of cell growth.

We next investigated the effects of RBR copy number reduction in the proximal meristem. Plants with reduced *RBR* copy number in significant portions of the meristem do not decrease root growth rate and meristem size up to 7 days after heat shock (data not shown). Clones generated in meristematic derivatives of stem cells after short heat shock corroborate that all cells outside of the root cap display wt growth characteristics upon RBR reduction (Figure 4I). Our data substantiate the specific effects on stem cell maintenance observed in the RNAi lines. Moreover, they indicate that columella cell growth is most sensitive to acute RBR reduction because it requires reduction of only one *RBR* gene

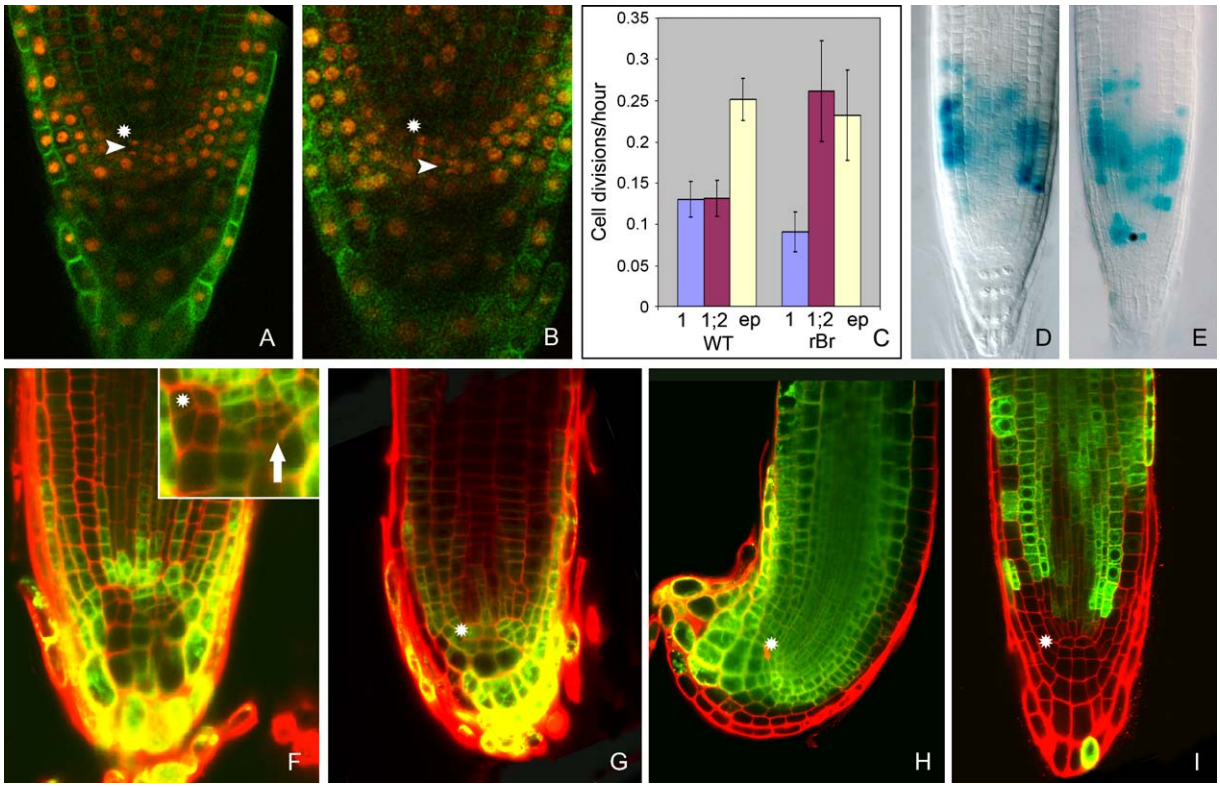

Figure 4. RBR Reduction Promotes Stem Cell Maintenance and Inhibits Cell Growth

(A–C) Time-lapse recording of metaphases in stem columella stem cell-like layers of rRBr plants. Division in layer immediately distal to the QC (A); division in a more distal layer (tier 2) (B); quantification of frequency of divisions in columella stem cell layer (1), columella layers 1 and 2 (1;2) and epidermal cells of proximal meristem (ep) (C). Error bars represent standard errors. Green, 35S::LPTI2:GFP marks plasma-membrane; red, 35S::H2B:YFP colocalizes with DNA.

(D and E) D Box CYCB1:1 GUS in wt (D) and rRBr (E) roots.

(F–I) Heat-shock-induced RBR reduction-of-function clones in *pCB1/pCB1,rbr3-1/+* plants. *rbr*$^{-}$ cells marked by GFP (green) and counterstained with propidium iodide (red); red-green overlap appears as yellow. Stem cell proliferation including GFP-negative stem cells (arrow) (F); stem cell proliferation and bending (G); bending only (H); proximal excisions without phenotype (I). Asterisk, QC.

copy. Proximal meristem cells only show expansion defects after simultaneous *RBR* reduction and mutation of the *SCR* gene. Our data suggest that RBR affects cell growth, as has been observed for *Drosophila* G1-S transition regulators (Johnston et al., 1999).

In one plant, we found supernumerary stem cells that had not undergone *RBR* excision themselves, but abutting cells that had undergone *RBR* excision (Figure 4F, inset). This observation suggests that *RBR* reduction may influence stem cell fate nonautonomously.

Inducible Overexpression of RBR Leads to Rapid Loss of Stem Cell Identity

To analyze whether downregulation of RBR is essential for stem cell maintenance, we overexpressed *RBR* cDNA fused to the GR domain for inducible activation. As negative control, we used RBR cDNA with an essential amino acid substitution (RBR^{c788p}; Huntley et al., 1998; Kaye et al., 1990). When seedlings were transferred on 0.1μM dexamethasone (dex), columella stem cell identity in *35S::RBR:GR* was lost within 1 day as differentiated cells adjoined the QC (Figures 5A and 5B) and ground tissue stem cell identity was compromised as judged by the loss of *At5g44160* RNA (Figures 5I and 5J). Both stem cell effects occurred prior to any other phenotypic changes. *SCR, SHR, PLT* promoter activity and the QC25 marker were not affected in *35S::RBR:GR* on 0.1μM dex, confirming that patterning is not primarily affected upon manipulation of RBR activity (data not shown).

The expression of *D Box CYCB1;1:GUS* in the proximal meristem was not altered after 1 day on 0.1 μM dex, confirming that stem cells respond more sensitively than the proximal meristem to changing levels in RBR expression (Figures 5E and 5F). Upon prolonged exposure to 0.1 μM dex and on 1μM dex, however, root growth is reduced, meristem size severely decreases, and expression of *D Box CYCB1;1:GUS* disappears (Figures 5C, 5D, 5G, and 5H).

The sensitive response of stem cells to RBR overexpression and the contrasting consequences compared to RBR reduction further support that modulation of RBR activity is critical for stem cell maintenance.

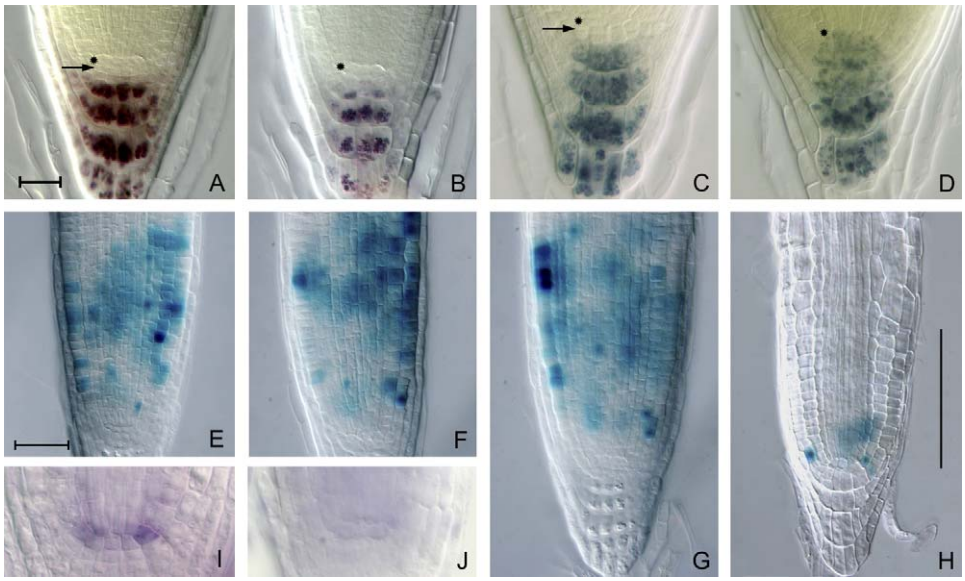

Figure 5. Induced RBR Overexpression Interferes with Stem Cell Maintenance

(A–D) 35S::RBR^{c788a}:GR (A and C) and 35S::RBR:GR (B and D) at 0.1 μM (A and B) and 1 μM (C and D) dexamethasone.

(E–H) D Box CycB1:1 GUS in 35S:: RBR^{c788a}:GR (E and G) and 35S::RBR:GR (F and H) at 0.1 μM (E and F) and 1 μM (G and H) dexamethasone.

(I and J) *At5g44160* mRNA in 35S::RBR^{c788a}:GR (I) and 35S::RBR:GR (J) at 0.1 μM dexamethasone.

DIC images after GUS stain (blue), starch granule staining (dark purple), or after in situ hybridization (light purple). Arrow, columella stem cells; asterisk, QC position; vertical bar, meristem size. Bar, 25 μm.

KRP2, CYCD3, and E2Fa Affect Stem Cell Fate in Accordance with Their Postulated Roles in the Plant RBR Pathway

According to current models of cell cycle regulation in mammals and plants, cyclin D (CYCD) members act upstream of RB and inhibit its activity. The root growth rate of *35S::CYCD3;1* overexpression lines are not changed compared to wt (Figure 2Q) but, like in rRBr roots, additional small cells are generated in the root cap stem cell area (Figure 6A). The columella accumulates undifferentiated cells (Figure 6A), and an additional LRC layer is generated in *35S::CYCD3;1* roots. Ablation of the cells at the QC position in *35S::CYCD3;1* roots leads to rapid differentiation of excessive undifferentiated columella cells, indicating that they are stem cells (Figure 6B).

In current cell cycle models, activity of RBR can be maintained when CDK/CYCD complexes are inhibited. KRPs are predicted to inhibit CDK/CYCD action in plants (De Veylder et al., 2001). We analyzed roots of a *35S::KRP2* overexpression line, and consistent with KIP action in an RBR stem cell pathway, stem cells are rapidly consumed. At 5 dpg, the columella stem cell region contains differentiated cells (Figure 6C) and the LRC contains one layer less than wt (Figure 6D). Furthermore, the cortex/endodermis stem cells are consumed and cells that are located at their position are frequently elongated (Figure 6D, arrowhead). Only after the effects on stem cell consumption appear, root meristem size and root growth rate decrease (Figure 2Q). Thus, overexpression of KRP2 mimics the effect of RBR overexpression on stem cell status.

RB binds and inhibits transcription factors of the E2F class (Weinberg, 1995). An excess of E2F might therefore also lead to supernumerary stem cells. Indeed, roots that express *35S::E2Fa* and its cofactor *35S::DPa* accumulate additional undifferentiated cells in the columella and LRC stem cell zone (Figure 6E). Ablation of the QC indicates that these extra cells are stem cells (Figure 6F).

To further investigate whether KRP2, CYCD, RBR, and E2F act in vivo in a common pathway, we investigated epistatic relationships. Combinations of stem cell-restricting *35S::KRP2* with stem cell-promoting *35S::CYCD3*, rRBr, and *35S::E2Fa/DPa* lines resulted in all cases in stem cell excess, consistent with CYCD, RBR, and E2F factors acting downstream of KRP activity in stem cell maintenance (Figures 6G–6L).

While our gain-of-function data cannot identify individual members of the KRP, CYCD, and E2F families that are influencing stem cell maintenance, they strongly suggest that RBR controls stem cell pool size in response to D-type cyclins and through the modulation of E2F action.

DISCUSSION

Retinoblastoma-Related Proteins as Common Stem Cell Regulators in Animals and Plants

Factors required for ''stemness'' in animal systems include signaling pathways that are utilized for other developmental decisions (Reya et al., 2003; Sancho et al., 2003; Hitoshi et al., 2002; Alonso and Fuchs, 2003; Lin, 2002; Song and Xie, 2003), intrinsic transcription factors to maintain stem cell

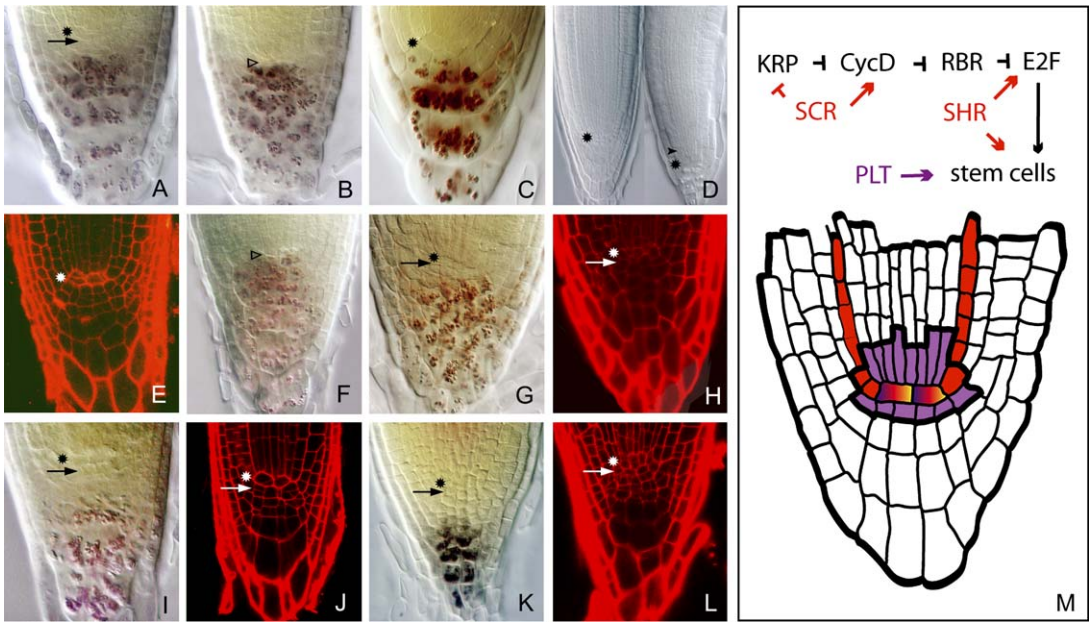

Figure 6. Genes in the Canonical RBR Pathway Affect the Root Stem Cell Pool

(A and B) 35S::CYCD3 with excessive stem cells before (A) and after (B) laser ablation.

(C and D) 35S::KRP2 roots with stem cell loss (C) compared to wt control on the left (D).

(E and F) 35S::E2Fa,35S::DPa roots with excessive stem cells before (E) and after (F) laser ablation.

(G and H) Combined 35S::CYCD3 and 35S::KRP2 overexpression results in restoration of stem cells.

(I and J) Combined 35S::E2Fa, 35S::DPa, and 35S::KRP2 overexpression results in excessive stem cells.

(K and L) Combined rRBr and 35S::KRP2 overexpression results in excessive stem cells.

Arrow, columella stem cells; asterisk, QC; open arrowhead, ablated QC remnants; closed arrowhead in (D), elongated cells at stem cell position. DIC images (A–D, F–G, I, and K); CLSM images (E, H, J, and L).

(M) A model for interactions between the stem cell-maintaining RBR pathway and patterning genes involved in stem cell specification. Schematic root shows expression domain of SHR and SCR in red and of PLT in purple. SCR action in stem cell maintenance is cell autonomous for the QC (yellow).

fate (Chambers et al., 2003; Mitsui et al., 2003), and translational repressors that may prevent differentiation (Tsuda et al., 2003; Hayashi et al., 2004; Wang and Lin, 2004; Chen and McKearin, 2005; Szakmary et al., 2005). Previously identified factors for stem cell maintenance in *Arabidopsis* influence stem cell fate through their upstream roles in cell specification. For example, the *WUS* gene in the shoot and the *SCR* gene in the root act within organizers for stem cells (Mayer et al., 1998; Schoof et al., 2000; Sabatini et al., 2003). The *PLT* genes also mediate root stem cell specification but are expressed in organizing as well as in stem cells (Aida et al., 2004). While niche organization is similar in both kingdoms, the putative transcription factors that specify the plant stem cell niche are plant specific, illustrating that stem cell specification evolved independently.

In contrast to the apparent divergence of stem cell fate regulators in plants and animals, our discovery that the *Arabidopsis* retinoblastoma-related protein (RBR) specifically regulates stem cell fate in the root is suggestive for common mechanisms of stem cell maintenance. How general is plant stem cell control by RB-related proteins? Local overexpression of RBR in *Arabidopsis* and tobacco suggests that RBR influences cell differentiation and the pool size of dividing cells also in the shoot apical meristem (J. Wyrzykowska, M. Schorderet, S. Pien, W.G., and A. Fleming., unpublished data; L.M., H. Feiler, J. Fütterer, A. Fleming, and W.G., unpublished data). Although it is not yet fully clarified whether RBR action specifically affects the shoot stem cell pool, the data suggest similar activities of RBR in roots and shoots of diverse plants.

There is mounting evidence for the relevance of RB in mammalian stem cell maintenance. Stem cell RNA profiling data reveal that CYCD (Ramalho-Santos et al., 2002) and genes annotated to encode RB binding and -inhibiting proteins (Ivanova et al., 2002) are elevated in mouse stem cells. High sensitivity to reduction of RB-like proteins in presumed stem cells has been observed in mice (Vanderluit et al., 2004; Ruiz et al., 2004; Sage et al., 2003; Ferguson et al., 2002), although definitive stem cell identification is difficult in these systems.

Since the upstream factors that control stem cell positioning in plants and animals evolved independently, it appears that RB regulation for stem cell maintenance has been ''recruited'' independently by distinct patterning gene cassettes. Alternatively, ancestral unicellular eukaryotes might have maintained cells with different proliferation competence, consistent with the recent discovery of proliferation capacity differences in unicellular organisms (Stewart et al.,

2005). An RB homolog with a G1 commitment role has been discovered in a single-celled alga (Umen and Goodenough, 2001). Thus, RB-mediated ''proliferation capacity'' control could have predated the divergence of unicellular plant and animal ancestors and become incorporated as ''stem cell maintenance'' control in both kingdoms.

Connection of RB-Mediated Stem Cell Regulation to Patterning Cues

Our study reveals that RBR reduction bypasses the requirement for the *SCR* patterning gene in stem cell maintenance. *SCR* is only required in the organizing QC cells for stem cell maintenance (Sabatini et al., 2003), indicating that its role is to downregulate the RB pathway in the QC or in stem cells. It is worth it to note that our QC ablation data reveal necessity of QC signaling in all backgrounds with reduced RBR pathway activity. This rules out that a SCR-controlled QC signal downregulates the RBR pathway in stem cells, as this would make RBR-reduced stem cells independent of the QC signal. In line with this interpretation, the RBR reduction clones suggest that stem cells may be affected non-cell-autonomously. Together, our observations support a scenario where RBR downregulation in the QC organizer is critical for stem cell control. Unambiguous evidence for the site of action of the RBR pathway will however require QC- and stem cell-specific elimination of RBR function.

Possible mechanisms by which SCR may maintain the stem cell state include local downregulation of KRPs or upregulation of D cyclins. While our epistasis analysis suggests that these factors can influence stem cell maintenance through the RB pathway, it is at present not clear which of the G1 regulatory components in these large protein families might be controlled by SCR. Preliminary results suggest complex changes in mRNA levels of several G1 but not G2 regulators in *scr* mutants indicating feedback control (J.M.P.-P. and B.S., unpublished data). In this context, it may be relevant that growth effects occur upon acute removal of a supernumerary copy of RBR, indicating homeostatic control of *RBR* signaling potentially mediated by feedback control on RNA and protein levels of G1 regulators.

Interestingly, the roles of SHR and PLT proteins in stem cell maintenance cannot be bypassed by *RBR* reduction, suggesting that they regulate other essential factors for stem cell status. Such independent input is consistent with the combinatorial fashion in which these proteins program the stem cell status together with SCR (Aida et al., 2004). These inputs may also impinge on the RB pathway (for example by regulating the local availability of factors acting downstream of RB such as E2Fs), as suggested by the epistasis of *plt1,2* double mutants over RBR reduction (Figure 6M). However, it cannot be excluded that they control yet to be discovered stem cell regulatory mechanisms that act in parallel.

RB-Mediated Stem Cell Maintenance: Regulation of Division or Differentiation?

Both cell cycle entry and regulation of differentiation-mediating factors are well-known RB functions in animal systems. For example, human E2Fs can induce cell cycle progression factors as well as cell differentiation factors (Muller et al., 2001).

In one scenario, premature G2 entry due to RBR reduction could speed up the cell cycle in stem cells resulting in differentiation delay. We found no evidence for changes in overall cell cycle length after RBR manipulation by our in vivo measurements of mitotic frequency. This may be explained by RBR functions separate from cell cycle control or by cell cycle length compensatory mechanisms such as those described in *Drosophila*, where shortening of one phase is accompanied by lengthening of another phase (Neufeld et al., 1998; Reis and Edgar, 2004).

In a second more plausible scenario, RBR reduction represses differentiation by potentiating a QC-related stem cell-promoting factor either in QC or in stem cells. In this model, RBR would directly influence cell differentiation. There are ample precedents for roles of RB-related proteins in cell differentiation (Liu et al., 2004), and RB can regulate targets unrelated to cell cycle progression (Ross et al., 2001). As QC-dependent stem cell proliferation in roots occurs upon overexpression of E2F transcription factors, candidate stem cell promoting factors may form a subset of E2F-regulated genes which can now be investigated in detail.

EXPERIMENTAL PROCEDURES

Plant Materials and Genetic Analysis

Markers, mutants, and transgenic lines are as follows: N9099 lateral root cap marker, the Nottingham Stock Center; QC25, QC46, and QC184 (Sabatini et al., 1999; Sabatini et al., 2003) ; *shr-1* (Benfey et al., 1993); *scr-4* (Fukaki et al., 1998); *SHR:GFP* and *SCR:GFP* (Nakajima et al., 2001); *pWER::GFP* (Lee and Schiefelbein, 1999); *pGL2::GUS* (Masucci et al., 1996)(*D Box CYCB1;1:GUS* (Colon-Carmona et al., 1999); *LTI6b:GFP* (Cutler et al., 2000); *H2B:YFP* (Boisnard-Lorig et al., 2001); *plt1-4*, *plt2-2* and *PLT1::GUS* (Aida et al., 2004); *35S::E2Fa*, *35S::DPa* and *35S::KRP2* (De Veylder et al., 2001); *35S::CYCD3;1* plants (Dewitte et al., 2003).

RBR RNAi

323 bp of the 5′ end of the *RBR* cDNA clone AF245395 was cloned in forward and reverse orientation, separated by a spacer. The resulting fragment was combined with the RCH1 promoter (Casamitjana-Martinez et al., 2003) in vector pCARi-323 and transformed into Col-0 plants. Twenty-two independent transformants were analyzed and contained identical phenotypes.

RBR Loss-of-Function Clones

A 8421 bp genomic *RBR* fragment, digested from BACF28J15 with ClaI and NheI and encompassing 1.5 kb upstream of the ATG to 2 kb downstream of the coding region, was cloned in between *lox* sites of pCB1 (Heidstra et al., 2004). The resulting construct pCB1-RBR was transformed into Col-0 plants and genotyped for a single insertion using a GFP probe. Three independent Col-0 lines containing the heat-shock-inducible Cre recombinase construct (Heidstra et al., 2004) were crossed with heterozygous *rbr1-3* plants (Ebel et al., 2004). Resulting F1 seedlings were crossed to pCB1-RBR homozygotes; progeny were genotyped for heterozygous T-DNA insertion, Norfluorazon-selected for pCB1, and selfed. Resulting plants were genotyped for homozygous RBR T-DNA insertion using a PCR fragment generated by primers CTGGAAAGCT GATGATAATGGTATAGAAGG and TATGGTGCAAGTGCAGGTTAGTTA ATTATG and by Southern blotting after NdeI digestion to discriminate between endogenous RBR and pCB1-donated RBR. Homozygotes for the

rbr1-3 allele were not recovered, but transmission of the *rbr1-3* allele through one of the gametes was restored to 100% (Tables S1 and S2). Furthermore, plants with two copies of endogenous RBR and two copies of pCB1-RBR were not recovered, indicating that the presence of four RBR alleles is lethal (Table S2). Genotyped F2 plants were selfed again; clones were induced by 20 or 60 min heat shock and recorded by confocal laser scanning microscopy. Only clones from *RBR*$^{+}$*/RBR*$^{+}$*,pCB1/Ø* and *rbr1-3/RBR*$^{+}$*,pCB1/pCB1* plants were analyzed, as copy number reductions could be derived in these backgrounds (Table S3). Clones generated in *RBR*$^{+}$*/RBR*$^{+}$ *pCB1/Ø* plants revealed root cap bending, due to acute reduction from three to two RBR copies. Root cap bending and stem cell defects or root cap bending alone was observed in clones from the *rbr1-3/RBR* + *pCB1/pCB1* background, indicating that reduction of the number of RBR copies from three to one was required for stem cell defects.

Conditional RBR Overexpression

RBR cDNA and a point mutation variant RBR^{c788p} obtained by PCR were cloned into the polylinker of the PTA7002 vector (Aoyama and Chua, 1997) and transformed into Col-0 plants (L.M., H. Feiler, J. Fütterer, A. Fleming, and W.G., unpublished data). Homozygous lines were selected. Independent transformants with the wild-type RBR cDNA gave rise to similar phenotypes on 0.1 and 1 μM dexamethasone.

Microscopy

Whole-mount visualization of roots, starch granule staining, and β-glucuronidase stains were done as in Willemsen et al. (1998). Whole-mount in situ hybridization was performed manually using a protocol described in Friml et al. (2003) with a 793 nt *RBR* cDNA fragment created using primers AGATGGCTTGACCTACTTTGAGGATTTAC and GAAACTCTCAATTAC CTTGCTGAGATCAA and a 1397 nt *At5g44160* fragment downstream from the predicted start codon using primers ATGACAAGTGAAGTTCTTC AAACAATCTCAAGTG and CCATCCATTGATAGACGATGGATGGCAC AACG. Measurements of root length, meristem size, and number of meristematic cells as in Sabatini et al. (2003). CLSM and QC laser ablations were performed on a Leica SP2 inverted confocal laser scanning microscope as in van den Berg et al. (1997). In vivo time lapse recording of cell division in roots was performed using an automatic tracking method (B. Garcia et al., 2004, LNCS, abstract).

Western Blotting

RBR was quantified by means of SDS-PAGE followed by Western blotting. Root tips from wt and mutant were homogenized in buffer; proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. Equal loading was confirmed by using Memcode Reversible Protein Staining Kit (Pierce). 1:5000 diluted polyclonal antibody raised against RBR, provided by Dr. L. Bako, was applied followed by a goat-anti chicken IgY coupled to HRP (Santa Cruz Biotechnology). Decorated proteins were visualized by Enhanced Chemo Luminescence (Amersham Biosciences). The specificity of the RBR antibody was tested by immunological staining with the preimmune serum (data not shown).

Supplemental Data

Supplemental Data include three tables and one figure and can be found with this article online at http://www.cell.com/cgi/content/full/123/7/1337/DC1/.

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