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RETINOBLASTOMA-RELATED PROTEIN controls the transition to autotrophic plant development

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SUMMARY

Seedling establishment is a crucial phase during plant development when the germinating heterotrophic embryo switches to autotrophic growth and development. Positive regulators of embryonic development need to be turned off, while the cell cycle machinery is activated to allow cell cycle entry and organ primordia initiation. However, it is not yet understood how the molecular mechanisms responsible for the onset of cell division, metabolism changes and cell differentiation are coordinated during this transition. Here, we demonstrate that the *Arabidopsis thaliana* RETINOBLASTOMA-RELATED protein (RBR) ortholog of the animal tumor suppressor retinoblastoma (pRB) not only controls the expression of cell cycle-related genes, but is also required for persistent shut-down of late embryonic genes by increasing their histone H3K27 trimethylation. Seedlings with reduced RBR function arrest development after germination, and stimulation with low amounts of sucrose induces transcription of late embryonic genes and causes ectopic cell division. Our results suggest a model in which RBR acts antagonistically to sucrose by negatively regulating the cell cycle and repressing embryonic genes. Thus, RBR is a positive regulator of the developmental switch from embryonic heterotrophic growth to autotrophic growth. This establishes RBR as a new integrator of metabolic and developmental decisions.

KEY WORDS: *Arabidopsis*, Retinoblastoma, *RBR*, *ABI3*, Polycomb, PRC2, Cell fate, Cell cycle, Differentiation, Seedling establishment, Embryo development, Tumor, Sucrose

INTRODUCTION

Development of multicellular organisms requires asymmetric cell division and cell differentiation to produce shape and cell specificity. The coordination of these events is achieved by intercellular, intracellular and external environmental signals. These signals are integrated to produce a decision: either the cell undergoes another round of cell cycle or it exits from the cell cycle and differentiates. One of these signal integrators is the mammalian retinoblastoma tumor suppressor pRB, which is known to be inactivated in the majority (>70%) of human tumors and many different tumor types (Knudsen and Knudsen, 2006). pRB is a negative regulator of the G1/S-phase transition (Goodrich et al., 1991; Lee et al., 1987) and interaction of pRB with cell cycle-promoting E2F/DP transcription factor heterodimers form repressive complexes that block E2F target gene activity required for entry into S phase. Growth-promoting signals result in the activation of CDK4/6-cyclinD and CDK2-cyclinE complexes, which phosphorylate pRB and thus release E2F/DP to allow progression of the cell cycle (for reviews, see Burkhart and Sage, 2008; van den Heuvel and Dyson, 2008). In addition to the role in cell cycle restriction and tumor suppression, retinoblastoma proteins have been shown to regulate genes involved in differentiation of certain mammalian cell types in tissue culture (Korenjak and Brehm, 2005); however, the significance of pRB for fate decisions in vivo has been shown only for bone and brown adipose tissues (Calo et al., 2010). Thus, the molecular mechanisms by which retinoblastoma-related proteins control cell lineage commitments in vivo are not known in most cases.

The presence of retinoblastoma orthologs in angiosperms (RETINOBLASTOMA-RELATED protein, RBR) (Durfee et al., 2000) offers the opportunity to study RBR functions in a novel developmental context different from animals. During development from an embryo to the adult plant, the sporophyte undergoes few key transitions. One crucial transition occurs during seedling establishment, when the embryo germinates and the plant seedling gains photosynthetic competence. At this transition, the developmental program switches from heterotrophic growth, which is characterized mainly by cell expansion and a metabolism based on stored nutrients, to photoautotrophic growth and metabolism, and activation of meristem activities. This transition and the factors directing gene expression changes are still poorly understood.

The *Arabidopsis* genome encodes one pRB homolog, RBR, which is required to arrest mitosis in the embryo sac (Ebel et al., 2004). Thus, *rbr* loss-of-function alleles are gametophytic lethal, which makes it difficult to study the role of RBR in cell cycle, cell differentiation and development in the sporophyte. To overcome this problem, virus-induced gene silencing (VIGS) targeted against RBR in tobacco and expression of viral proteins known to interact with RBR in *Arabidopsis* have been used (Desvoyes et al., 2006; Jordan et al., 2007; Lageix et al., 2007; Park et al., 2005). These studies demonstrate the importance of *RBR* in restricting cell proliferation in leaf and stem tissues late in plant development. Similar phenotypes have been observed in plants that overexpressed simultaneously E2Fa and DPa transcription factors

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(De Veylder et al., 2002). RBR has also been implicated in the maintenance of 'stemness', because a local reduction of RBR in the root apical meristem leads to an increased number of stem cells (Wildwater et al., 2005). A recent study, using inducible RNA interference against *RBR*, demonstrated that RBR is a crucial regulator of stem cell proliferation in every stem cell niche, suggesting that stem cell maintenance is a general function of RBR (Borghi et al., 2010). However, the molecular mechanism underlying this remains unknown.

Most previous plant studies have focused either on the gametophyte (Chen et al., 2009; Ebel et al., 2004; Ingouff et al., 2006; Johnston et al., 2010; Johnston et al., 2008; Jullien et al., 2008) or on late sporophytic developmental stages (Borghi et al., 2010; Desvoyes et al., 2006; Johnston et al., 2010; Jordan et al., 2007; Lageix et al., 2007; Park et al., 2005; Wyrzykowska et al., 2006), and the function of RBR during developmental transitions has not yet been addressed. Embryo development in seed plants consists of a morphogenesis and a maturation phase. Seed maturation depends on the import of sugar and other nutrients for the synthesis and accumulation of storage macromolecules (Braybrook and Harada, 2008). These seed reserves are rapidly mobilized during seedling establishment (Tsukagoshi et al., 2007). Important regulators of late seed development include the B3 domain transcriptional repressors *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *LEAFY COTYLEDON2* (*LEC2*), *FUSCA3* (*FUS3*), *VP1/ABI3-LIKE1* (*VAL1*), *VAL2* and *VAL3* (Romanel et al., 2009). Overexpression of *ABI3* in seedlings led to high expression of seed storage proteins and to mutants strongly affected in accumulation of seed storage proteins (Nakashima et al., 2006). *VAL1* and *VAL2* have recently been identified as preventing sugar-inducible expression of seed maturation genes and play an essential role in regulating the transition from seed maturation to seedling growth (Tsukagoshi et al., 2007). Many transcription factors that regulate seed maturation and germination also play a role in abscisic and gibberellic acid signaling, but relatively little is known about how the transition from the germinating seedling to photoautotrophic growth and development is connected to the cell cycle and controlled by hormones.

In this study, we generated *Arabidopsis* lines with reduced levels of RBR early in sporophyte development. Mutant seedlings were developmentally arrested after germination and are hypersensitive to sugar stimulation, which resulted in ectopic callus-like cell proliferation that occurred regardless of the presence of supplied plant hormones. This demonstrated a role for RBR not only in cell cycle control, but also in regulating the transition to photoautotrophic plant growth and development. This transition is linked to RBR-mediated repression of a sucrose-inducible embryonic program that is mediated via activating POLYCOMB REPRESSIVE COMPLEX 2 (PRC2)-mediated H3K27 trimethylation of late embryonic genes. Thus, RBR links a metabolic and developmental transition in cell cycle control with PRC2-mediated gene repression.

MATERIALS AND METHODS

Plant material and growth conditions

For construction of *35S:RBR* and *35S:RBRi*, the plasmid pK7WG2 (Karimi et al., 2002) was modified for CaMV 35 promoter driven expression of a STREP-tagged (Schmidt and Skerra, 2007) genomic RBR gene or a RBR RNA hairpin as follows: for production of *35S:RBRcs* lines, the *Hin*dIII (position 3753) to *Xba*I (position 11871) fragment of pK7WG2 was replaced by 420 bp of the CaMV 35S promoter and a cassette containing 4863 bp of the genomic sequence of the *Arabidopsis RBR1* gene from start to stop codon of the reading frame. This was fused at its N terminus to a sequence coding for a STREP-tag [adapted from Schmidt and Skerra (Schmidt and Skerra, 2007) with the sequence MANWSHPQFEKGP] and followed by the bidirectional polyadenylation signal from ACMV (Bieri et al., 2002); following this RBR expression cassette, the DsRED expression cassette under control of the Napin promoter as described by Stuitje et al. (Stuitje et al., 2003) was inserted as a *Kpn*I-*Kpn*I fragment to allow visual selection of primary transformants.

For *35S:RBRi*, the genomic RBR sequence from the *Eco*RI site 1132 bp downstream of the start codon to the end was replaced by the RBR cDNA sequence from the start codon to this *Eco*RI site in antisense orientation. For *RBRi* cloning procedures, see Borghi et al. (Borghi et al., 2010). For production of *RBRcs* seedlings, the sequence upstream of *RBR* (until the transcription start of the next gene) and the sequence downstream of *RBR* (until the transcription start of the next gene) were fused to the complete coding sequence of *RBR* in the binary vector pCambia1300 [cloning performed according to Sambrook and Russell (Sambrook and Russell, 2006)]. *clf-29* and *swn-3* alleles are described elsewhere (Bouveret et al., 2006; Chanvivattana et al., 2004). For transformation of *Arabidopsis* (Columbia-0 accession), the floral dip method (Zhang et al., 2006) was used. T1 seeds were either selected via fluorescence of the seed coat or on Murashige and Skoog (MS) medium containing 50 μg/ml kanamycin, and after two weeks the seedlings were transferred to soil. Subsequent generations were grown in growth chambers (mixed fluorescent and incandescent light 150 μmol m^{-2} s^{-1} for 16 hours at 22°C). Seeds were sterilized according to standard methods, and stratified for 4 days at 4°C in the dark on MS growth medium with either 1% sucrose or equivalent-molar amounts of mannitol as control.

Molecular analysis

Immunoblot analysis, RNA extraction, RT-PCR, q-PCR and data analysis was performed as previously described (Borghi et al., 2010).

Microarray hybridization and evaluation

RNA from 3-day-old seedlings and three biological replicates were labeled, hybridized and measured on ATH1 microarrays (Affymetrix, Santa Clara, CA) as described (Hennig et al., 2004). Signal values were derived using the GCRMA algorithm implemented in R (R Development Core Team, 2009). Significance of differentially expressed genes was detected based on the rank-product algorithm (Breitling et al., 2004). Genes were considered as differentially expressed if $P<0.05$ and there was at least a twofold change. Significance of overlaps of gene sets was calculated with a hypergeometric distribution test. PCA analysis was performed with MEV (http://www.tm4.org/), using the full algorithm and standard settings. Cluster analysis was carried out with the Genevestigator tool (http://www.genevestigator.ethz.ch) (Hruz et al., 2008) and with MEV.

Protein detection with mass-spectrometry

Proteins (50 μg) were subjected to SDS PAGE and in-gel digestion was performed (see Shevchenko et al., 1996). Mass spectrometry measurements were performed on an LTQ FT-ICR (Thermo Finnigan), coupled with a Probot (LC-Packings/Dionex) autosampler system and an UltiMate HPLC-system (LC-Packings/Dionex) as described previously (Agne et al., 2010). MS/MS spectra were searched with TurboSequest and PeptideProphet by using the Trans-Proteomic Pipeline (TPP v2.9) against the *Arabidopsis* TAIR8 protein database (downloaded on 14th December 2007) supplemented with contaminants. The search parameters were: requirement for tryptic ends, one missed cleavage allowed and mass tolerance=±3 Da. Carbamylation of cysteine was set as fixed, and modification and oxidation of methionine was set as a variable modification. For PeptideProphet, the cutoff was set to a minimum probability of 0.9; APEX factors were determined as described previously (Lu et al., 2007).

Histological analysis and cell counting

Tissues were fixed in ethanol:acetic acid (9:1). After dehydration (90% to 70% ethanol scale for 1 hour each) tissues were cleared in clearing solution [66.7% (w/v) chloral hydrate and 8.3% (w/v) glycerol in water]. Images were recorded with an AxioCam HRc CCD camera and epidermal cells were counted along the length of the hypocotyls of at least 10 mature embryos or seedlings from *RBRcs* and wild type. Alternatively, embryos were DAPI

stained and observed with a Zeiss Axioplan microscope. GUS staining was performed as described previously (Brand et al., 2002). Fat-red staining was performed as described previously (Tsukagoshi et al., 2007).

Scanning electron microscopy

For scanning electron microscopy, a CamScan CS-44 microscope (emitter: LaB6, detector systems: EDX, Orientation Imaging Microscopy) was used together with provided software. Prints of the analyzed *Arabidopsis* tissues were obtained following the previously published protocol (Kwiatkowska, 2004).

Cytological analysis

Ploidy analysis was performed with 1-week-old etiolated seedlings according to the manufacturer's instructions (Partec, Münster, Germany) with a Partec Ploidy Analyzer. For quantification, the results of two independent preparations were averaged.

Fluorescence measurements

Fluorescence measurements were performed with a Closed FC 800-C (Photon Systems Instruments) fluorcam with the provided software. For measurement, seedling containing plates were dark-adapted for 20 minutes and the appropriate program from the software package was started. For analysis, data from at least 10 seedlings per time point and genotype were used.

Assay of sugar concentrations

Aliquots (about 20 mg fresh weight) of frozen tissue powder were extracted with ethanol, and sucrose, glucose and fructose levels were determined (see von Schaewen et al., 1990). The insoluble pellet was used for determination of starch (see Hendriks et al., 2003).

Chromatin immunoprecipitation (ChIP)

For ChIP analysis, 0.2 g plant material (FW) per sample was used. Preparation of plant material and formaldehyde crosslinking were performed as previously described (Bowler et al., 2004). Isolation and sonication of chromatin, and immunopreciptiation, elution and reverse crosslinking of chromatin were performed with the LowCell#ChIP kit from Diagenode (Diagenode, Liège, Belgium) according to the manufacturer's instructions. For immunoprecipitation, affinity-purified α-RBR antibodies and anti-trimethyl-histone H3 Lys 27 antiserum (Millipore, catalog #07-449) were used. Immunoprecipitated DNA was analyzed by quantitative PCR as described above and the tested regions were within 500 bp upstream of the translation start site of the respective gene. For quantification, results from two PCR reactions were used and displayed as percent of input. All ChIP experiments were performed with at least two biological replicates.

Sequences of primers used for cloning, quantitative RT-PCR and ChIP can be found in Table S8.

RESULTS

Mutant seedlings with reduced RBR levels are developmentally arrested after germination

To examine the role of *RBR* during early seedling growth, we used three different approaches to downregulate RBR protein activity. Transformation of wild-type (wt) *Arabidopsis* with a construct for constitutive expression of *RBR* RNAi (*35S:RBRi*) or with constructs for expression of *RBR* under the control of the *35S* promoter (*35S:RBR*) resulted in seedlings that were developmentally arrested after germination (see Fig. S1 in the supplementary material). As it was not possible to select mutant seedlings with standard-resistance markers, we used a fluorescent marker (*DsRED*) under the control of a seed-specific promoter for selection of transgenic plants (Stuitje et al., 2003). Seedlings containing either of these constructs showed reduced RBR protein levels (see Fig. S1B in the supplementary material); thus, the *35S:RBR* transgene caused RBR co-suppression instead of RBR overexpression. Additionally, we obtained several independent homozygous lines expressing the *RBR* cDNA under the control of a 2.1 kb *RBR* promoter fragment (*RBRp:RBRcDNA*). These lines segregated in the T2 and later generations (see Table S1 in the supplementary material) for seedlings developing normally and for seedlings arrested with the same phenotype as *35S:RBRi* seedlings (Fig. 1A-D). Arrested seedlings showed reduced RBR levels (Fig. 1J) and were named *RBR* co-suppression (*RBRcs*). Phenotypically normal seedlings showed elevated RBR protein levels (Fig. 1J) and were named RBR overexpression (*RBRoe*). This allowed us to investigate seedlings with strongly reduced RBR levels in more detail because we were not restricted to T1 seedlings, as in the case

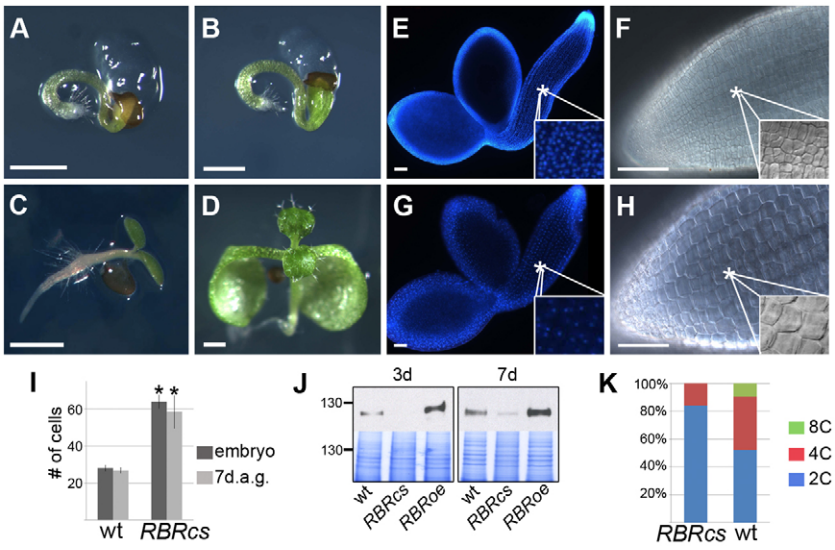

Fig. 1. Reduction of RBR protein causes embryonic cell proliferation and seedling arrest. (**A-D**) *RBRcs* seedlings germinate (3 d.a.g., A) but arrest after expansion (7 d.a.g., B; 3- and 7-day-old wild-type seedlings in C and D). Scale bars: 1 mm. (**E-H**) Additional cell divisions in *RBRcs* (E,F) versus wild type (G,H) revealed by DAPI staining (E,G) and differential interference contrast (F,H). Scale bars: 50 μm. (**I**) Epidermal cell numbers along embryonic and seedling hypocotyls (I: **P*<0.01). (**J**) Immunoblot analysis of 3-day- and 7-day-old wild-type, *RBRoe* and *RBRcs* seedlings. (**K**) *RBRcs* seedling cells accumulated with 2C DNA content and showed less endoreduplication (DNA content >4C).

of the *35S:RBRi* and *35S:RBR* lines. All mutant seedlings with reduced RBR levels, irrespective of the approach, had identical phenotypic abnormalities (Fig. 1A-D; see Fig. S1 in the supplementary material), whereas *RBRoe* seedlings continued development without obvious phenotypic abnormalities. Beginning at the bent cotyledon stage, *RBRcs* embryos could be distinguished by their increased cell number (Fig. 1E-I; P=1.1E-13). Embryo morphology and cell specification were normal, as shown by the typical cell file pattern of the *GL2::GUS* reporter (see Fig. S1K in the supplementary material). Shoot apical meristem development was arrested, however, and *CLV3* expression was absent (see Fig. S1L in the supplementary material). After germination, *RBRcs* seedlings remained in an embryonic state and retained their apical hook and closed cotyledons (Fig. 1A-D). The majority of cells retained a 2C genome content (Fig. 1K), suggesting that they were arrested in G1. Together, these results show that *Arabidopsis* RBR is required during embryogenesis to restrict cell proliferation and to support development after germination.

Expression profiling reveals upregulation of cell cycle-specific genes in *RBRcs* mutant seedlings

To understand the molecular basis of the developmental arrest, we performed a microarray experiment with RNA from 3-day-old *RBRcs* mutant and wild-type seedlings. We detected 1872 and 1939 differentially up- and downregulated genes (P<0.05, fold change ≥2; see Table S7 in the supplementary material for the whole dataset) in *RBRcs* mutants, demonstrating the importance of RBR for maintaining gene expression homeostasis. Clustering of our data set with genes deregulated in mutants ectopically expressing *E2Fa/DPa* (Vandepoele et al., 2005) revealed a highly significant overlap of gene expression changes (Fig. 2A), showing that RBR, together with E2Fa/DPa, acts in the same pathway to regulate gene expression. In addition, many genes with a typical M-phase and S-phase expression pattern (Menges et al., 2003) were strongly upregulated in *RBRcs* mutants (Fig. 2B; see Table S2 in the supplementary material). When all genes significantly upregulated in *E2Fa/DPa* mutants or with a canonical E2F-binding site in their promoter (Vandepoele et al., 2005) were removed from our data set, genes with M- but not with S-phase-specific expression were still significantly enriched among the deregulated genes in *RBRcs* seedlings. This suggests that RBR regulates genes for S-phase progression via the E2Fa/DPa pathway, but the expression of many genes during mitosis is independent of E2Fa/DPa transcription factors.

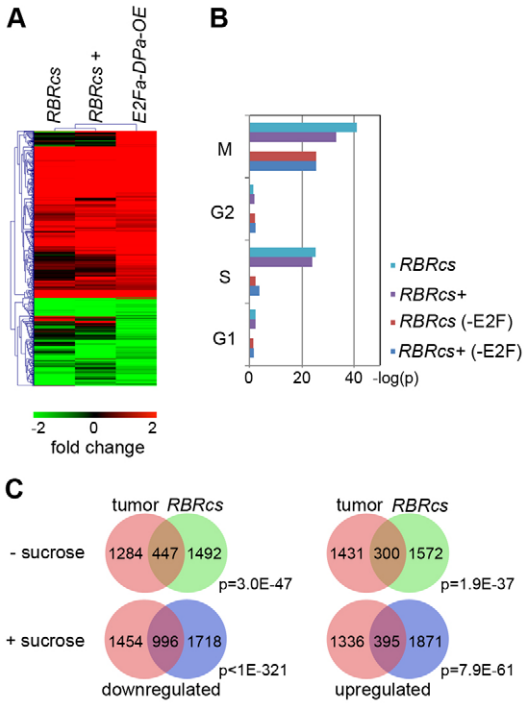

Fig. 2. Gene expression analysis reveals new RBR functions. (**A**) Clustering of 632 genes with significantly changed expression (P<0.05 and fold change >2) in 6-day-old *E2Fa-DPa-OE* seedlings (Vandepoele et al., 2005) shows highly significant correlation in gene expression with *RBRcs* mutants grown with (*RBRcs+*) or without (*RBRcs*) sucrose (P-value for the number of overlapping genes in *RBRcs* and *E2Fa-DPa-OE*: without sucrose, 2.18E-212 for upregulated and 5.72E-94 for downregulated genes; with sucrose, 1.09E-223 for upregulated and 1.62E-82 for downregulated genes). (**B**) Genes upregulated in *RBRcs* mutants are significantly enriched for S- and M-phase-specific genes. M-phase-specific genes are still significantly enriched if all genes with elevated expression in *E2Fa-DPa-OE* and containing E2F sites (Vandepoele et al., 2005) in their promoter are removed. Shown are negative $\log_{10}$ values of the likelihood to obtain an observed fraction of cell-cycle phase-specific genes by chance. For gene numbers, see Table S2 in the supplementary material. (**C**) Venn diagram representation of significantly up- and downregulated genes in *RBRcs* mutant seedlings and *Agrobacterium*-induced tumors (Deeken et al., 2006). P-values were calculated with the hypergeometric test.

RBRcs seedlings are physiologically a sink tissue

Interestingly, the RNA profile of *RBRcs* seedlings also showed a significant overlap with gene expression changes in *Agrobacterium*-induced tumors (Deeken et al., 2006) (Fig. 2C). Unlike transformed mammalian cells, plant cells do not usually proliferate into tumors, except in response to altered plant hormone regimes induced by specialized pathogens such as *Agrobacterium* (Inze and De Veylder, 2006). *Agrobacterium*-induced tumors become a sink tissue that relies on carbon sources provided by photosynthetic tissues (Deeken et al., 2006). This observation led us to consider a possible role for RBR in metabolic regulation during germination and transition from heterotrophy to autotrophy. During germination, proteins and neutral lipids previously stored in the embryo are metabolized (Fait et al., 2006; Penfield et al., 2006). Subsequently, seedlings acquire photosynthetic capacity, which supports autotrophic growth and post-embryonic development (Bentsink and Koornneef, 2008; Graham, 2008; Santos-Mendoza et al., 2008). Upregulation of nine sugar transporters, reduced expression of key photosynthetic genes and reduced chlorophyll fluorescence (see Tables S3, S4 and Fig. S2A in the supplementary material) indicated that *RBRcs* seedlings retained a sink tissue identity with compromised transition to photoautotrophic growth.

RBRcs seedlings strongly respond to sucrose

To examine whether *RBRcs* seedling development might be arrested due to a metabolic restriction, we provided germinating seedlings with a carbon source. Addition of 1% (28 mM) sucrose to the growth medium dramatically changed the phenotype of *RBRcs* mutant seedlings but had no visible effects on wild-type seedlings (Fig. 3A-G). Seven days after germination (d.a.g.), cotyledons of *RBRcs* mutants opened, 60% of the *RBRcs* seedlings developed short roots, and all showed ectopic cell division activity (Fig. 3E-G). In some cases (<2% of seedlings), holes developed in cotyledons, probably because of disrupted cell-cell contacts (Fig. 3C). In ~30% of *RBRcs* seedlings, outgrowth of misdifferentiated

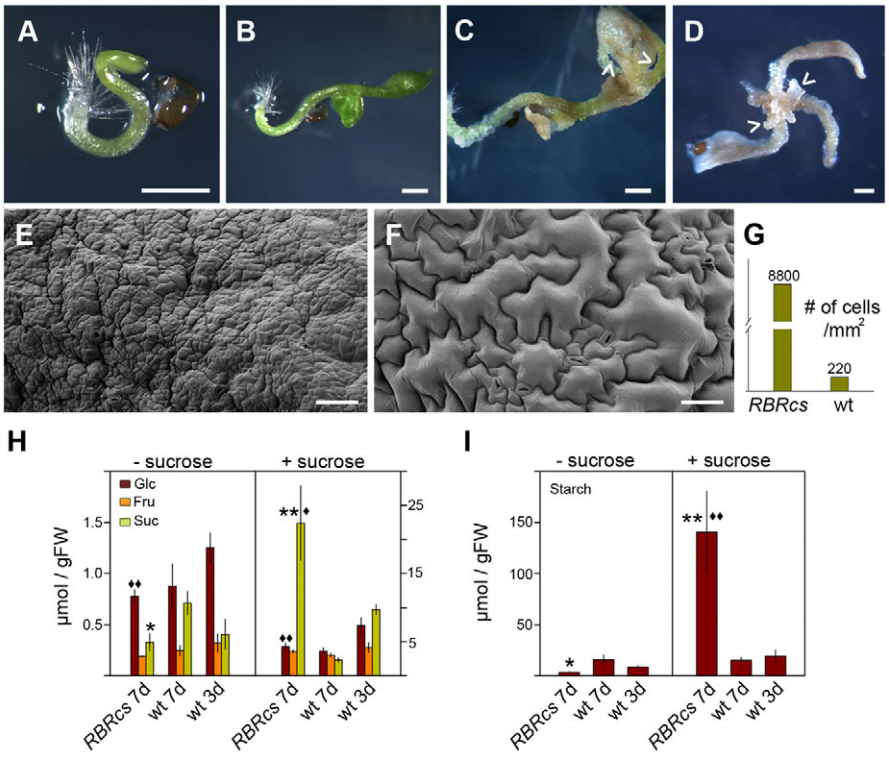

Fig. 3. Sucrose induces tumor-like, heterotrophic growth in *RBRcs* mutant seedlings. (**A-D**) Ectopic cell division in *RBRcs* mutants grown in the presence of 1% sucrose for 3 (A), 7 (B), 14 (C) and 21 (D) days. Occasional development of holes in *RBRcs* cotyledons (C, arrowheads) and outgrowth of structures from the shoot apex (D, arrowheads) are observed. Scale bars: 1 mm. (**E**,**F**) SEM pictures and epidermal cell number (**G**) of the adaxial side of cotyledons of 14-day-old seedlings grown on sucrose. Scale bars: 50 µm. (**H**,**I**) Without sucrose, 7 day-old *RBRcs* seedlings contained less sucrose, glucose and starch than wild-type seedlings. With sucrose, *RBRcs* seedlings accumulated significantly more sucrose and starch than all controls (harvest at the end of day, $P<0.05$ (single diamond), $P<0.01$ (double diamond) compared with 3-day-old wild-type seedlings; $*P<0.05$, $**P<0.01$ compared with 7-day-old wild-type seedlings).

structures at the site of the shoot apical meristem suggested a resumption of meristem activity (Fig. 3D). Although the expression profile of cell cycle-related genes in *RBRcs* seedlings was not strongly altered by sucrose (Fig. 2B; see Table S2 in the supplementary material; total number of genes with deregulated expression in *RBRcs* mutant seedlings grown on sucrose: 2266 upregulated and 2450 downregulated, $P<0.05$, fold change ≥2; see Table S7 in the supplementary material for the whole dataset), the overlap with the tumor expression profile increased (Fig. 2C). The proportion of cells with a 4C DNA content increased (see Fig. S2B in the supplementary material), suggesting that DNA replication was affected by the energetic status of *RBRcs* seedlings or by sucrose signaling. The high levels of sucrose and starch in *RBRcs* seedlings grown on sucrose (Fig. 3H,I) further suggested a strong physiological shift towards heterotrophic growth, which was also supported by a principal component analysis (PCA) of the gene expression data (Fig. 4).

Sucrose causes a shift towards embryonic identity in *RBRcs* mutant seedlings

The PCA (Fig. 4) also indicated that growing *RBRcs* seedlings on sucrose had caused a shift to the embryonic gene expression program. In order to determine which genes may be responsible for the sugar response, a hierarchical clustering of the 100 most strongly induced genes in *RBRcs* mutants grown on sucrose (see Table S5 in the supplementary material) was performed using the Genevestigator anatomy tool (Zimmermann et al., 2004). From these 100 genes, a cluster of 67 genes is specifically expressed in seeds, imbibed seeds, endosperm and embryos (Fig. 5A). None of these genes changed expression in response to 1% sucrose in wild-type seedlings and most were not deregulated in *RBRcs* seedlings grown without sucrose (see Fig. S3A in the supplementary material). To confirm the accumulation of the respective gene products, proteins from 3-day-old *RBRcs* and wild-type seedlings grown on medium with 1% sucrose were extracted and subjected to SDS page. After tryptic digest and subsequent tandem mass spectrometry, we were able to obtain quantitative information for each identified protein by calculating an Absolute Protein EXpression factor (APEX) (Lu et al., 2007). The APEX factor is calculated by normalizing the number of identified spectra of each protein to its theoretical number of tryptic peptides and to the size of each dataset (i.e. the total number of identified spectra; see Table S7 in the supplementary material for the whole dataset). We identified 33 proteins that were encoded by the above identified embryo-specific expressed genes. All showed a higher abundance in *RBRcs* in comparison with wild type with respect to total number of tryptic peptides and APEX factor (Fig. 5B; see Table S6 in the supplementary material).

Consistent with the observed derepression of late-embryonic specific genes in *RBRcs* mutants grown on sucrose, we detected in *RBRcs* seedlings an accumulation of neutral lipids usually present only in wild-type mature embryos. In *RBRcs* seedlings grown on sucrose, we found accumulation of neutral lipids in cotyledons already 7 days after germination and a conspicuous staining in 14-day-old seedlings (Fig. 5C-F).

The strongly upregulated genes in *RBRcs* seedlings included the transcription factors *ABI3* and *ABI5*. *ABI3* activates seed maturation genes, acts upstream of *ABI5* (Lopez-Molina et al., 2002) and binds to the Sph/RY (CATGCA) motif (Carranco et al., 2004; Monke et al., 2004; Reidt et al., 2000; Suzuki et al., 1997). This motif was significantly enriched in promoters of the 100 strongest upregulated genes in *RBRcs* seedlings on sucrose medium (motif occurrence within 500 bp upstream of the start-codon: 57 times, $P=5.26E-9$). *val1/val2* double mutants are seedling lethal and show a strong derepression of embryonic genes as a consequence of ABI3, LEC2, FUS3 or LEC1 overexpression (Suzuki et al., 2007; Tsukagoshi et al., 2007). *VAL1* and *VAL2* expression was not changed in *RBRcs* seedlings (see Fig. S3B in the supplementary material), but *ABI3* and

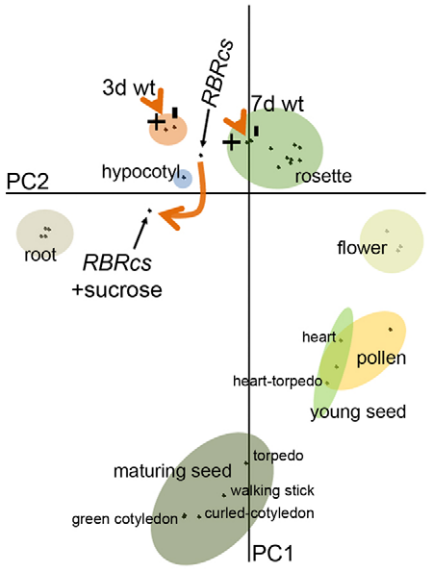

Fig. 4. Sucrose shifts gene expression in *RBRcs* seedlings to a late seed development program. Principal component analysis (PCA) of 1137 genes (5% of genes present on the Affymetrix ATH1 array) that show the highest expression variation among different *Arabidopsis* tissues (Schmid et al., 2005) and *RBRcs* versus control seedlings grown with and without sucrose. The first principal component accounted for 37% of the variation in the data and separated photosynthetic from sink tissues. The second principal component accounted for 22% of the variation in the data. Addition of sucrose caused only a minor shift in gene expression in 3- and 8-day-old wild-type seedlings (arrowheads) but a conspicuous shift towards maturing seeds in the first principal component and roots in the second principal component in *RBRcs* mutant seedlings (red arrow; +, with sucrose; –, without sucrose).

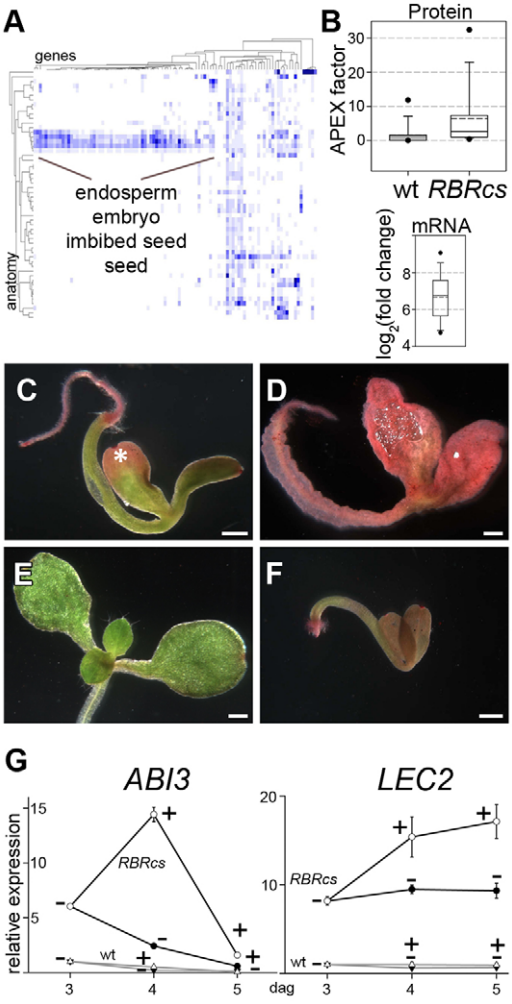

Fig. 5. Sucrose induces embryonic traits in *RBRcs* mutants. (**A**) Sixty-seven of the 100 most strongly induced genes in *RBRcs* mutants (see Table S5 in the supplementary material) cluster with embryo- and seed-specific genes. (**B**) Box plot representations of the APEX factor for 33 seed-specific proteins that could be detected by MS/MS in *RBRcs* seedlings (dots indicate the 95th and 5th percentiles, broken line indicates the mean value) and the $\log_2$ of their expression changes (for details see Table S6 in the supplementary material). (**C-F**) Sudan red staining reveals accumulation of lipids in *RBRcs* seedlings grown on sucrose (C, 7 d.a.g.; the asterisk marks the beginning of lipid accumulation; D, 14 d.a.g.) compared with wild type on sucrose (E, 7 d.a.g.) and *RBRcs* without sucrose (F, 14 d.a.g.). Scale bars: 1 mm. (**G**) *LEC2* and *ABI3* expression responded to sucrose in the *RBRcs* mutant but not in wild-type seedlings when germinated on medium without sucrose and 3 d.a.g. transferred to medium either with 1% (+) or without (–) sucrose. See also Fig. S3 in the supplementary material.

LEC2 were strongly induced by sucrose in 3 d.a.g. *RBRcs* but not in wild-type seedlings (Fig. 5G; see Fig. S3B in the supplementary material). In addition, there was a significant overlap of upregulated genes in *val1/val2* and *RBRcs* seedlings grown on sucrose (see Fig. S3C in the supplementary material).

In order to distinguish whether the induction of this embryonic program was a direct effect of RBR downregulation or caused indirectly by the ectopic cell division activity in *RBRcs* seedlings, we tested embryonic gene expression levels in a previously reported *Arabidopsis* line that was transgenic for β-estradiol inducible RNAi against RBR (*RBRi*) (Borghi et al., 2010). Seedlings grown in the dark elongate without significant cell division (Gendreau et al., 1997). Therefore, we germinated *RBRi* and wild-type seeds on estradiol and on plates with and without sucrose for 2 days in the dark and then analyzed expression of embryonic genes in the dark and 1 day after de-etiolation in the light. As for the *RBRcs* seedlings, RNA levels for the transcription factors ABI3 and LEC2, and the seed storage protein CRU3 were upregulated in the etiolated *RBRi* but not wild-type seedlings. A light-dependent plastid marker (LHCB) remained downregulated after transfer to light (see Fig. S4 in the supplementary material).

Together, these results suggested that RBR acts as a repressor of the sugar-inducible embryonic transcriptional program and hence as a regulator of autotrophic seedling establishment. This RBR regulatory function is required during germination and independent of an earlier function of RBR in controlling the rate of cell division during late embryogenesis.

RBR binds directly to promoters of embryonic genes and is required for H3K27 trimethylation to establish permanent silencing after germination

To understand the mechanism by which RBR might regulate the switch from heterotrophic embryo to autotrophic seedling development, we considered the possibility that RBR may inactivate late embryogenesis genes in concert with POLYCOMB REPRESSIVE COMPLEX 2 (PRC2), which in animals and plants establishes histone H3K27me3 marks to repress developmental

genes (for reviews, see Hennig and Derkacheva, 2009; Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). The expression of *CURLY LEAF* and *SWINGER*, two methyltransferases that are active in PRC2 in *Arabidopsis* seedlings (Chanvivattana et al., 2004), was not reduced in *RBRcs* mutants (see Fig. S5A in the supplementary material); however, the expression of *ABI3*, *LEC2* and *CRU3* was strongly increased and was sucrose inducible in *clf swn* double mutants (see Fig. S5B in the supplementary material). Analysis of our microarray data for genes enriched in H3K27me3 in mature plants (Zhang et al., 2007) revealed a significant overlap with the 100 most differentially expressed genes in *RBRcs* seedlings grown on sucrose (Fig. 6A). This significant overlap was not present when we used the whole dataset of upregulated genes, but it was even more pronounced in the cluster of embryonic/seed-specific genes (31 out of 67 with H3K27me3, $P<9.77e-10$) and included genes encoding sugar transporters, seed storage proteins, *LEC2* and *ABI3*. We selected *ABI3*, *LEC2*, *RESPONSIVE TO ABA 18* (*RAB18*), *LATE EMBRYOGENESIS ABUNDANT1* (=*GEA1*), *At3g54940* (annotated as cysteine-type endopeptidase), *CRUCIFERIN 3* (*CRU3*) and *SUCROSE TRANSPORTER 4* (*SUT4*) for a kinetic analysis of H3K27 trimethylation. In wild-type seedlings, H3K27me3 levels increased up to 50-fold in the chromatin of these genes at 7 d.a.g. (Fig. 6B). By contrast, their H3K27m3 modification in *RBRcs* seedlings remained at low levels, consistent with their persistent activity. H3K27me3 levels in *AGAMOUS* and *SEPALLATA3* (Fig. 6B; see Fig. S5C in the supplementary material), which regulate flower development (Honma and Goto, 2001; Mizukami and Ma, 1992), remained high and unchanged in wild-type and in *RBRcs* seedlings, demonstrating that *RBRcs* seedlings are not generally defective for H3K27me3 chromatin modification. Involvement of RBR in inactivation of the genes most likely to be regulated by PRC2 was further supported by αRBR ChIP-experiments, which showed that RBR interacts with the promoters of *RAB18*, *GEA1*, *SUT4*, *ABI3* and *CRU3* (Fig. 6B; see Fig. S5C in the supplementary material). RBR was not found on the promoters of *LEC2* and At3G54940, suggesting that in these genes RBR might bind more distantly from the transcriptional start site or that their inactivation by RBR may be indirect or require additional factors. These data support the conclusion that RBR facilitates seedling establishment by repressing promoters of late embryogenesis genes during germination.

DISCUSSION

We modulated RBR protein levels early during seedling development by various methods. Two of them (*35S:RBR* and *RBp:RBRcDNA*) resulted in RBR co-suppression. The third approach (*35S:RBRi*) induced RNA interference against *RBR* and therefore it is very unlikely that the phenotypes we observed in *RBRcs* lines were due to an initial transient *RBR* upregulation. We obtained several independent *RBRp:RBRcDNA* lines that segregated reproducibly into RBR overexpressors (*RBRoe*) and RBR co-suppressors (*RBRcs*), which allowed us to analyze the effect of RBR downregulation, despite of the complete growth arrest. Although seedlings with increased levels of RBR did not show any discernable phenotype, siblings with co-suppressed *RBR* expression and reduced protein levels revealed an unexpected role for RBR during germination and seedling establishment.

During embryogenesis, *RBRoe*, *RBRcs* and wild-type embryos were phenotypically indistinguishable at early developmental stages. This suggests that either RBR levels are not crucial at these stages or that RNAi or co-suppression mechanisms are not yet effective, although it has been reported that components of the silencing machinery are important early during embryo development (Kerstetter et al., 2001; Lynn et al., 1999; Schauer et al., 2002). From the bent cotyledon stage onwards, embryos that later became arrested during seedling development had an increased number of cells. Additional cells in *RBRcs* embryos may be the result of a shortening of the cell cycle or a prolongation of the proliferative phase (De Veylder et al., 2002). However, it appears that these embryonic cells arrested in G1, because after germination and release from potential growth-restrictions by the seed coat, no further ectopic cell division or an increase in DNA

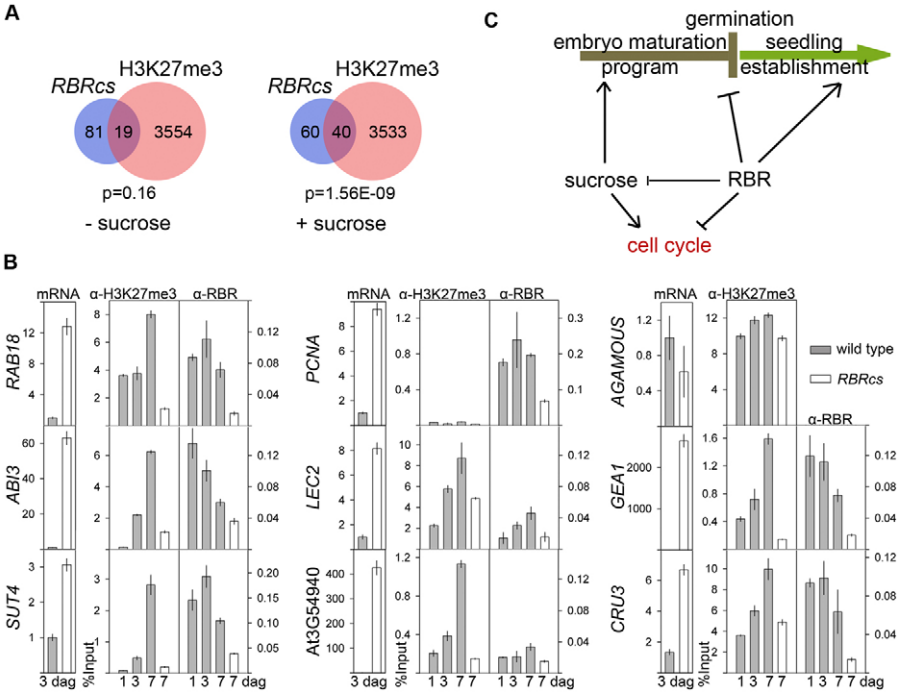

Fig. 6. RBR binds to promoters of genes that become repressed via H3K27me3 during seedling establishment. (**A**) Venn diagram of genes marked by H3K27me3 in mature plants and the 100 genes most strongly upregulated in *RBRcs* ±sucrose. (**B**) Kinetic analysis of H3K27me3 and RBR promoter interaction by ChIP. Methylation of seed-specific genes increases gradually for 7 d.a.g. in wild type but much less in *RBRcs*, while methylation of control genes *AGAMOUS* and *PCNA* is not differentially affected. RBR binds to the promoters of *ABI3, RAB18, GEA1, SUT4* and *At3g54940*. Gene profiling data were confirmed by quantitative PCR for these genes (mRNA panels). (**C**) Model for the function of RBR and sucrose during embryo maturation and seedling establishment.

content could be detected during seedling expansion. This is in contrast to the reported increase in cell division and/or DNA endoreplication associated with RBR downregulation or E2F/DP overexpression in older plants (De Veylder et al., 2002; Park et al., 2005) and the reported G2 phase cell cycle arrest for RBR-depleted MM2d cells (Hirano et al., 2008). It appears that the effect of RBR interference depends on the developmental and/or metabolic state of the cell and possibly also on the mode of interference. The observed arrest of cells in G1 after ectopic divisions during *RBRcs* embryo development suggests that, besides RBR, at least one additional mechanism must exist to restrict cell cycle activity during late embryo development and early seedling establishment. Because the G1 block can be relieved by addition of 1% sucrose, it is possible that cells in *RBRcs* embryos proliferate as long as they are provided by sugars from maternal tissues. Taken together, RBR is not the only repressor of the G1/S transition or the sole target of sucrose-induced cell cycle initiation via activation of D-type cyclins, as previously proposed (Riou-Khamlichi et al., 1999; Riou-Khamlichi et al., 2000; Soni et al., 1995). Our data suggest that sucrose acts on alternative pathways that are necessary for cell cycle entry and meristem activity.

Different to the situation in animals, plants consist of source tissues that produce and export sugars such as the rosette leaves in *Arabidopsis*, and sink tissues that import sugars such as embryos, roots and the shoot apical meristem. Sucrose is not only the transportable form of the chemically fixed energy but also signals between these two different types of tissue to modulate growth and development (Rolland et al., 2006). Here, we show that RBR is an important regulator of the sugar response in germinating *Arabidopsis* seedlings. Future work will determine whether RBR is a direct target of sugar signaling pathways or if the effects of sucrose and RBR are mediated by the energy status of the seedling. In *Ostreococcus*, the metabolic status is signaled via cAMP, which results in the activation of a CyclinA/CDKA complex that inhibits RBR and allows entry into S-phase (Moulager et al., 2010). However, a possible role of cAMP in nutrient signaling in *Arabidopsis* remains to be identified (Gehring, 2010). Interestingly, in *Arabidopsis* cell suspension culture, RBR was found to be associated with S6K1 (Henriques et al., 2010), a growth regulator downstream of TOR kinase. Additional experiments will reveal whether the interaction of RBR with the TOR pathway also determines developmental decisions.

Seedlings with reduced RBR levels showed a hypersensitive response to sucrose and behaved in many respects like tumor tissue: sugar-transporters were upregulated in combination with highly increased sucrose accumulation, photosynthetic capacity and expression of genes of the photosynthesis machinery was reduced, and the cell division arrest was relieved. In normal plant development, the heterotrophic metabolism is associated with maturing embryos, roots and meristems, and the setup of metabolic pathways in these tissues may vary depending on their context. Plant tumors require imported sugars, and accumulation of sugar and starch in *RBRcs* seedlings grown on sucrose suggests that *RBRcs* cells also take up and store sugars more effectively than wild-type cells. Additionally, we showed that *SUT4* is directly regulated by RBR. SUT4 is a low affinity-high capacity sucrose transporter and seems to be necessary for phloem-loading of sucrose in source tissue (Weise et al., 2000). However, it is strongly expressed in sink tissue and could be important for sucrose uptake and determining sink strength (Weise et al., 2000). This could suggest that RBR is involved in the regulation of sucrose accumulation in developing seeds. Many deregulated genes in *RBRcs* seedlings that are involved in cell cycle and DNA metabolism are most certainly controlled through the interaction of RBR with E2F/DP. These genes are also rapidly induced in older plants after downregulation of RBR (Borghi et al., 2010) and thus seem to be controlled independently of the developmental stage. By contrast, only a few embryo-specific and photosynthetic genes are affected when RBR is downregulated later in plant development, suggesting that control of gene expression by RBR is integrated with development-specific programs. We show here that RBR is involved in the control of genes that regulate seed maturation and early germination, and that RBR is directly associated with at least some of the respective promoter regions. Thereby, RBR regulates the switch from late embryogenesis to autotrophic seedling development by repression of sucrose-inducible embryonic traits in seedlings (Fig. 6C). RBR may facilitate this transition by directly repressing promoters of late embryogenesis genes during germination, and subsequently recruiting PRC2 to maintain their inactive state after seedling establishment by H3K27me3 modification. Alternatively, RBR and PRC2 may act in concert to regulate the developmental switch, similar to the interaction of RBR and PRC2 during *Arabidopsis* gametophyte development (Johnston et al., 2008). In accordance with this model, double mutants of *curly leaf/swinger* also showed accumulation of seed storage-specific triacylglycerols (Aichinger et al., 2009) and upregulation of genes expressed specifically in the embryo.

The transcriptional repression of the embryonic program appears to be crucial around 3 d.a.g. Until then, the embryonic program can be reinstated in response to unfavorable environmental conditions or experimentally by feeding seedlings with high concentrations of sucrose (Lopez-Molina et al., 2001; Rook and Bevan, 2003). Our data suggest that this re-establishment of the embryonic program is possible because H3K27me3 modification of the late embryo-specific genes is still low after germination and increases only later during seedling development. Such a regulation may be advantageous for the developing seedling as long as hypocotyl elongation is fueled by reserves from the endosperm (Penfield et al., 2004) and the germinating seedling still has a heterotrophic metabolism. Alternatively, this regulation could allow survival in an extended quiescent state under unfavorable environmental conditions, as suggested by the presence of genes for production of osmoprotectants in the late embryonic gene expression profile (Lopez-Molina et al., 2001).

It is possible, however, that reduction of RBR in *RBRcs* plants may also affect other genes that are required during seedling development and that have not been identified in this study. For example, these would include genes that link the metabolic program of the seedling to the activities of the shoot and root apical meristems, which also require RBR (Borghi et al., 2010; Wildwater et al., 2005). A possible role for animal pRB in control of cell differentiation events that are linked to nutritional signaling was recently reported (Annicotte et al., 2009; Dasgupta and Milbrandt, 2009). Our data suggest that sugar signaling in plants also involves RBR and that the effects of sugar on cell cycle and on the switch from heterotrophic to autotrophic metabolism are regulated by RBR (Fig. 6C). An attractive hypothesis is that sugar signaling (among other activities) primes cells for division, whereas RBR ensures that the transition is unidirectional by inactivating late embryo-specific genes.

Together, our results provide new insights to uncover the network in which RBR establishes a node that connects cell cycle, genetic and metabolic programs during plant development.

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Competing interests statement
The authors declare no competing financial interests.

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