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FUNCTIONAL CHARACTERIZATION OF THE *ARABIDOPSIS* RETINOBLASTOMA RELATED PROTEIN

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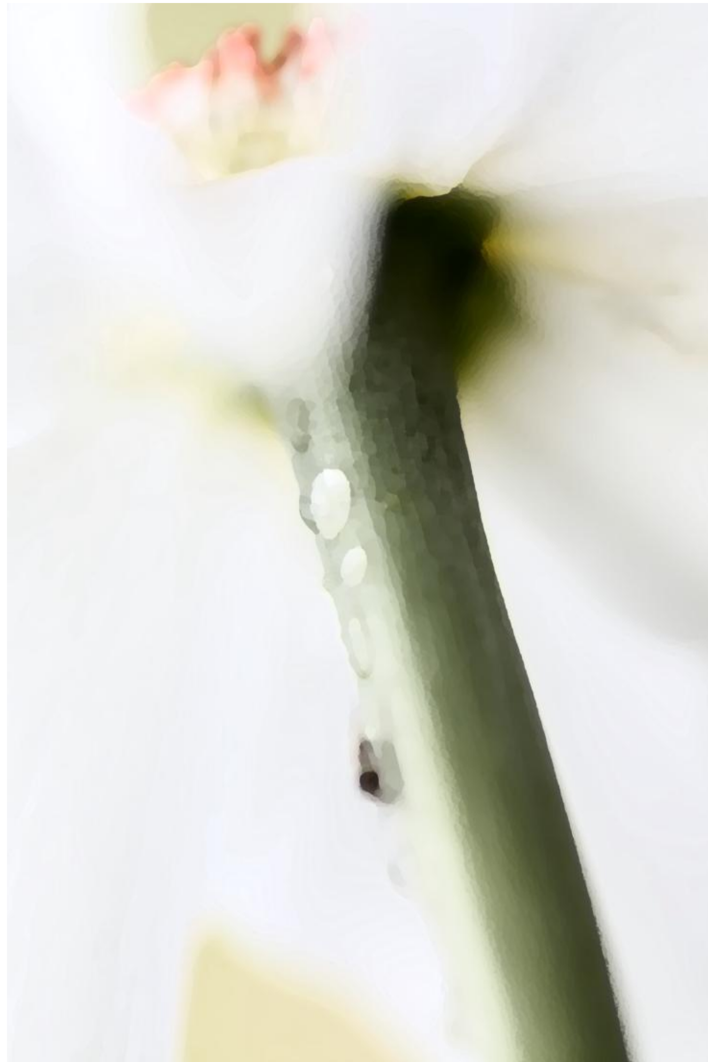

Abstract

The retinoblastoma protein (pRB) is a master regulator of cell cycle and differentiation in animal cells and as such an important tumor suppressor. It regulates the G1/S-phase transition via binding to E2F/DP transcription factors and therefore inhibiting expression of S-phase genes. Some studies provide evidence that pRB is also important for cell fate determination. However, whether this is an effect only on some specialized cell types or if pRB has a general effect on cell differentiation remains unresolved.

The presence of retinoblastoma-related proteins (RBRs) in plants offers the opportunity to study the function of this protein in a completely different developmental context. For example, plant organs develop after embryogenesis, plants switch from heterotrophy to autotrophy during germination and plants do not develop tumors without infection of specialized pathogens.

RBR knockout alleles are gametophytic lethal, which makes it difficult to study the role of RBR for cell cycle, differentiation and development in the sporophyte. Therefore we generated *Arabidopsis* mutant lines that displayed reduced expression of *RBR (RBRcs*-mutants). Mutant embryos showed a strongly increased number of cells in all organs but developed otherwise relatively normal. However, after germination *RBRcs* mutants were developmentally arrested and were not able to switch from an embryonic heterotrophic growth to an autotrophic seedling growth. *RBRcs* seedlings were able to efficiently take up sucrose, which resulted in massive ectopic cell over-proliferation. Gene profiling revealed a strong derepression of embryonic and seed maturation genes in the presence of sucrose. Our results suggest that 1) *Arabidopsis* seedlings require RBR to become autotrophic after germination and 2) RBR is required for repression of sucrose inducible embryonic and seed maturation genes after germination; thus RBR connects cell fate switch in seedlings after germination with cell cycle control.

Furthermore we generated antibodies against RBR and established co-immuno-precipitation assays to identify potential interaction partners of RBR. Subsequent MS/MS based analysis revealed that RBR binds to all three Arabidopsis E2F and both DP transcription factors. We also found potential new interactions and could accurately map several post-transcriptional modifications of RBR. This enabled us to propose a RBR interaction network, which could be an important resource for future research.

Finally we found an intriguing phenomenon. Seedlings with reduced activity of RBR always showed increased levels of *RBR* transcript. The same was the case for genes necessary for transcriptional gene silencing and DNA *de novo* methylation. We provide evidence that RBR is functionally connected with the RNA interference/transgene silencing pathway and seems to have a role in regulating certain genes on a posttranscriptional level.

Zusammenfassung

Das Retinoblastoma protein (pRB) ist ein wichtiger Zell-Zyklus Regulator und als solches ein wichtiger Tumor-suppressor in Säugetierzellen. Es inhibiert E2F/DP Transcriptionsfaktoren welche notwendig sind um S-Phase gene zu induzieren und reguliert so den G1/S-Phase Uebergang. Einige Studien konnten auch zeigen, dass pRB wichtig ist für Zelldifferenzierungsvorgänge. Ob diese Effekte nur in bestimmten Zell-typen wichtig sind, oder ob pRB eine mehr generelle Rolle in Zelldifferenzierung hat ist noch nicht eindeutig geklärt.

Die Tatsache, dass Planzenzellen ebenfalls Retinoblastoma-homologe besitzen (RBRs) bietet die Möglichkeit, die Funktion dieser Proteine in einem völlig neuen Entwicklungsbiologischen Zusammenhang zu studieren. Zum Beispiel entwickeln sich Pflanzenorgane erst *nach* der Embryogenese, Pflanzen ändern ihren Metabolismus von heterotroph zu autotroph nach der Keimung und Pflanzen entwickeln keine Tumore (mit Ausnahme wenn sie von spezialisierten Pathogenen infiziert werden).

Da *RBR*-Null *Arabdiopsis* mutanten schon als Gametophyten absterben ist es schwierig den Einfluss von RBR auf Zell-Zyklus-Kontrolle, Zell-Differenzierung und Entwicklung im Sporophyten zu erforschen. Deswegen entwickelten wir *Arabidopsis* Mutanten die weniger RBR produzierten (*RBRcs*-Mutanten). Embryonen dieser Mutanten entwickelten sich relative normal, zeigten aber eine grössere Anzahl Zellen im Vergleich zum normalen Phänotyp. Nach der Keimung waren *RBRcs* Mutanten in ihrer Entwicklung arretiert und konnten keinen autotrophen Sämling etablieren. Jedoch konnten *RBRcs* Mutanten sehr effizient Saccharose aufnehmen und zeigten unter diesen Bedingungen dramatische Zellteilungs-Aktivität. Das Expressionsprofil von *RBRcs* Mutanten zeigte, dass Samen-und Embryo-spezifische Gene stark durch Saccharose reaktiviert wurden. Unsere Ergebnisse legen nahe, dass 1) *Arabidopsis*-Sämlinge RBR benötigen um nach der Keimung Autotroph zu werden und 2) RBR Samen-und Embryo-specifische Gene nach der Keimung reprimiert. Das bedeutet, dass RBR Zell-Differenzierungvorgänge mit Zell-Zyklus-Kontrolle in jungen Keimlingen verbindet.

Ausserdem produzierten wir hoch-spezifische Antikörper gegen RBR und konnten damit ein Co-Immuno-Präzipitierungs Protokoll entwickeln. Damit war es möglich mit Hilfe von Massenspektrometrischen Methoden Interaktionspartner von RBR zu identifizieren. In diesen Assays interagierte RBR mit allen drei in *Arabidopsis* vorkommenden E2F und beiden DP transcriptionsfactoren. Ausserdem fanden wir zahlreiche neue Interaktionspartner und Post-Transkriptionelle Modifizierungen von RBR. Mit diesen Erkenntissen war es möglich ein hypothetisches RBR-Interaktions-Netzwerk zu postulieren welches eine wichtige Quelle für künftige Studien sein könnte. Schliesslich entdeckten wir ein interessantes Phänomen; Sämtliche *Arabidopsis* Mutanten mit

reduzierter RBR Aktivität zeigten stets eine höhere Expression von RBR auf Transkriptionsebene. Das gleiche Phänomen fanden wir für Gene die eine wichtige Rolle in RNA Interferenz und DNA-Methylierung haben. Daraus schliessen wir, dass RBR funktionell mit RNA Interferenz und DNA-Methylierung verknüpft ist und auch auf Post-Transkriptioneller Ebene die Aktivität von bestimmten Genen regulieren kann.

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1. Introduction

1971 Dr. Alfred Knudson published a statistical analysis of retinoblastoma and proposed that it arises after occurrence of two genetic events (Knudson 1971). More than a decade later it was hypothesized that these “two hits” result in a biallellic loss of a tumor suppressor (Cavenee et al. 1983; Godbout et al. 1983). In the late 80s the retinoblastoma gene has been identified by three different groups (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987) and its tumor suppression activity was validated. The RB protein is inactivated in more than 70% of human tumors in a wide range of tumor types (Knudsen and Knudsen 2006). RB became one of the most studied single proteins in clinical research and deciphering its mode of action in the context of tumor development an important topic in cell biology. This is illustrated by more than 15000 entries for the search term retinoblastoma in Pubmed and a new publication referring to retinoblastoma appeared every second day during the last four years. At the latest since its interaction with E2F transcription factors has been revealed (Chellappan et al. 1991) and further its importance in regulating G1/S-phase transition, it has become clear that pRB could also be a major player in developmental processes. This connection of pRB, cell cycle and differentiation on one hand and growth and development on the other has become of increasing interest in developmental and cellular biology and an increasing number of model organisms are used to track down how pRB functions.

On the origin of retinoblastoma-related proteins

If the function of retinoblastoma-related proteins (RBR) had to be described in a single word, “break” would probably be most appropriate. In a sense of being a break in the cell cycle and regulating whether a cell is allowed to enter a new round of cell division. Such a cell cycle checkpoint ensures that the initiation of one step is dependent upon the completion of the prior step which could be for example cell size or completion of DNA replication (Elledge 1996). The ability to restrain cell division when lacking sufficient nutrients exists in all living organisms.

The cell cycle of bacteria consists of overlapping, parallel processes in which individual steps appear to be only loosely linked together (Haeusser and Levin 2008) (Nordstrom et al. 1991). An elegant solution of how to connect nutrient availability to cell size control has evolved in *Bacillus subtilis*. Here an enzyme involved in glucolipid biosynthesis, UgtP, has been co-opted as a metabolic sensor. UgtP localizes to the division site in a glucose dependent manner and inhibits assembly of the tubulin-like cell division protein FtsZ (Weart et al. 2007). This ensures that cells reach an appropriate mass prior to initiating

cytokinesis. Defects in UgtP result in the formation of unusually small daughter cells and defects in chromosome segregation (Weart et al. 2007). Interestingly in the asymmetric, polarized eubacterium *Caulobacter crescentus* a host of checkpoints are included in the cell cycle (Goley et al. 2007). This could suggest that asymmetry of an organism increases the selective pressure for cell cycle checkpoints to evolve.

Since the cell cycle of eukaryotes and eubacteria is so profoundly different it is probably not surprising that RBR homologs have not been found in eubacteria. The cell cycle of Archaea especially of crenarchaea displays several eukaryotic-like features such as a clear distinction of G1, S, G2 and M-phase (Lundgren and Bernander 2005), multiple origins of replication (Lundgren et al. 2004; Robinson et al. 2004) and a 10-fold lower replication rate (Lundgren et al. 2004). A recent study provided a genome-wide transcription map of the cell cycle of the hyperthermophilic crenarchaeon *Sulfolobus acidocaldarius* (Lundgren and Bernander 2007). The existence of a *CDC6*-like gene and genes encoding proteins with a cyclin-box fold (Noble et al. 1997) in this organism may suggests similar regulatory features as eukaryotic cyclin/CDK complexes. A protein that is induced in *Sulfolobus* mitosis has similarity to eukaryotic p60 katanin protease which is involved in disassembly of microtubules (Baas et al. 2005).

Another report suggested that the B-domain of RBR may be derived from an archaeote ancester and the A-domain from a poxvirus before these genes fused in an ancestral eukaryote and gave rise to the *RBR* gene (Takemura 2005). However, this hypothesis should be treated with caution since the evidence is based only on very weak sequence similarities.

Due to the experimental limitations with extremophilic model-organisms our understanding of the archeal cell cycle is still in its invancy. But it is advancing at a rapid pace and might yield insights into the evolution of the eukaryotic cell cycle (Lundgren and Bernander 2005) and maybe the retinoblastoma-related protein.

In basal eukaryotes retinoblastoma-like proteins are not present. However, in budding yeast, *Saccharomyces cerevisiae* an RBR analog has been identified. The whiskey 5 mutant (*whi5*) is a small cell size mutant in budding yeast with a shortened G1 phase. WHI5 represses the yeast E2F analog SBF and is target of the yeast cyclin-dependent kinase (Costanzo et al. 2004). In contrast to the RBR/E2F regulatory circuit WHI5 is phosphorylated at all stages of the cell cycle and specific CDK sites function in WHI5 inactivation and regulation of cell size (Wagner et al. 2009). Despite these subtle differences WHI5 and RBRs serve as an excellent example of convergent evolution and attest the logic of this circuitry (Schaefer and Breeden 2004).

Amongst the more advanced eukaryotes, *RBRs* have been found in all members of the plant and animal lineage, in a ciliate and in *Dictyostelium*. This somewhat irregular distribution might show that *RBR* has been dispensable in many organisms and may

have been substituted by other checkpoint control mechanisms. Perhaps it's essential function in many organisms evolved only after plants and animals became multicellular and at that time it has been lost in fungi already. Indeed the RBR-homolog MAT3 from the unicellular green algae *Chlamydomonas reinhardtii* is – just as WHI5 in budding yeast – important for cell size control.

The structure and function of RBR proteins in several unicellular and multicellular organisms and the apparent cooption of RBR for new functions as control of differentiation and development will now be discussed in more detail.

Structural features of retinoblastoma-like proteins

Basically all the structural information about retinoblastoma-related proteins comes from the human retinoblastoma-related protein pRB. Due to the high sequence similarity of retinoblastoma-related proteins in plants and animals (about 40% sequence identity) it can be inferred that what has been found for the human pRB is also valid for plant RBRs. pRB is a nuclear phosphoprotein of around 100kDa in size. Purified pRB contains three structural, protease resistant domains (Figure 1) (Hensey et al. 1994). The N-terminal domain constitutes most of the aminoterminal half of pRB and is about 40kDa in size. The A and the B-domains have molecular masses of approximately 24 and 20kDa respectively (Goodrich 2003). The A and B domains are the most highly conserved regions of pRB throughout evolution. The A domain, a spacer region and the B domain are called the "small pocket" and most naturally occurring pRB mutants map to that region (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990). The B-domain contains also the so called LxCxE binding cleft, which mediates binding to cellular and viral proteins with a LxCxE motif.

When fused to a heterologous DNA binding domain the small pocket is sufficient to repress transcription (Sellers et al. 1995; Weintraub et al. 1995). Together with the ca 15kDa C-terminal region of pRB the small pocket can mediate full growth inhibition (Hiebert et al. 1992; Qin et al. 1992).

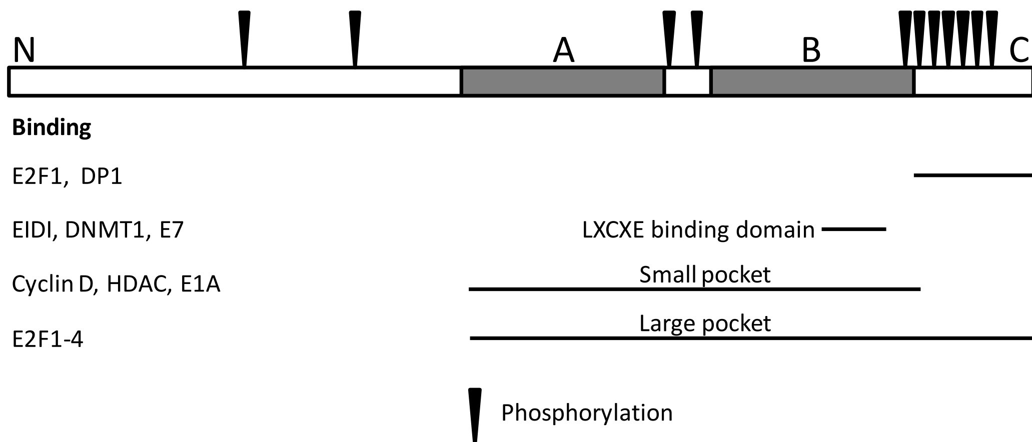

Figure 1: pRB structure and binding partners. Human pRB consists of three protease resistant domains, the N-terminal, A and B domain and does not contain commonly recognized DNA-binding or protein-interacting domains. Most binding partners bind to the so called A-B pocket region, which comprises also the LxCxE binding domain.Phosphorylation of RB enables cell cycle progression and occurs through releasing E2F transcription factors from the large pocket. After (Burkhart and Sage 2008).

In 1998 the first high-resolution structure of the small pocket bound to a viral LxCxE peptide has been published (Figure 2) (Lee et al. 1998). This study revealed that the A and the B domain both have a cyclin fold with highest conservation of residues at the interface between them and at the LxCxE binding site on the B-domain. Structurally, the binding of the B-domain to the LxCxE motive resembles the binding of cyclinA to the PSTAIRE helix of Cdk2 (Lee et al. 1998) (Jeffrey et al. 1995). Another important group of proteins that interact with retinoblastoma-related proteins are the E2F and DP class of transcription factors. Retinoblastoma-related proteins interact with E2F/DP heterodimers to repress transcription of E2F regulated genes which are often required to start the DNA-synthesis phase of the cell cycle (Dyson 1998). A structure with atomic resolution of an 18 amino-acid long peptide from E2F showed that E2Fs bind at the interface of the two lobes of the A/B-domain (Xiao et al. 2003). Furthermore this binding does not seem to induce structural changes in pRB. In the same study the authors showed that the peptide of the CR2 domain of the E7 protein of human papillomavirus and which binds via an LxCxE motive can reduce the binding affinity of a longer fragment of E2F to pRB. That solved the contradictory problem that the E2F/Rb complex is disrupted by E7 but that on the other hand E7 binds to a motive more than 30 angstrom apart.

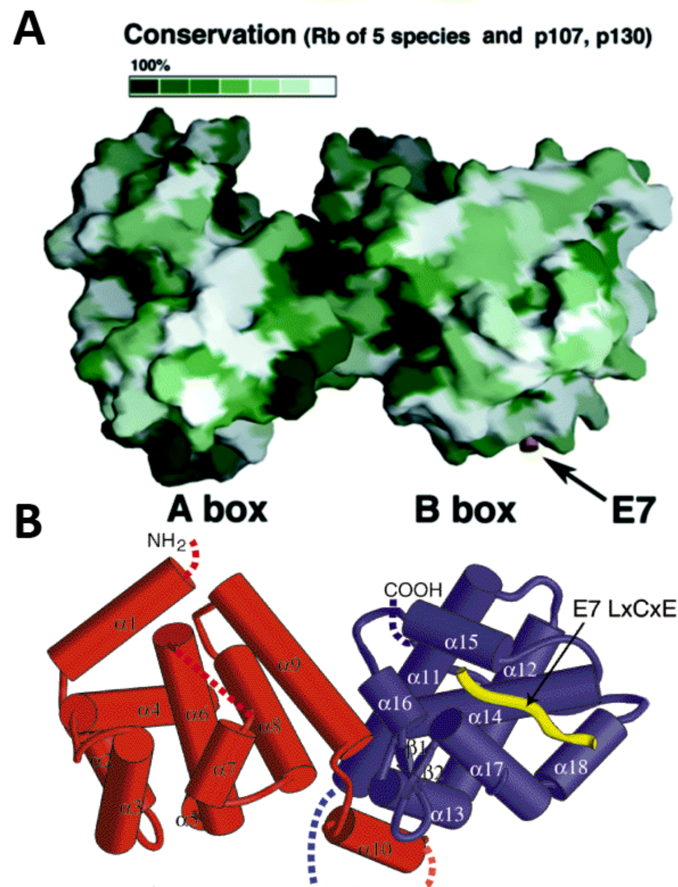

Figure 2: Structure of the pRB small pocket in complex with a LxCxE peptide.

A: The surface of the pocket is colored according to residue conservation across Rb homologues of five species. E2F transcription factors bind to the surface between the A and B domain and residues in this binding cleft are highly conserved.

B: Overall view of the Rb pocket bound to a LxCxE peptide (view is flipped vertically 180° compared to A). Dashed lines indicate regions that are disoredered in the structure . From (Lee et al. 1998).

A more recent structure showed that the CR1 region of the similar oncoprotein E1A of adenovirus binds to the same surface of pRB as E2F (Liu and Marmorstein 2007) (Figure 3). This could suggest a model for maximal E2F displacement from pRB by E1A. According to that E1A binds via the CR2 domain on the LxCxE binding site. CR2 is connected via a long flexible linker to CR1 which can now displace E2F and tightly bind to pRB.

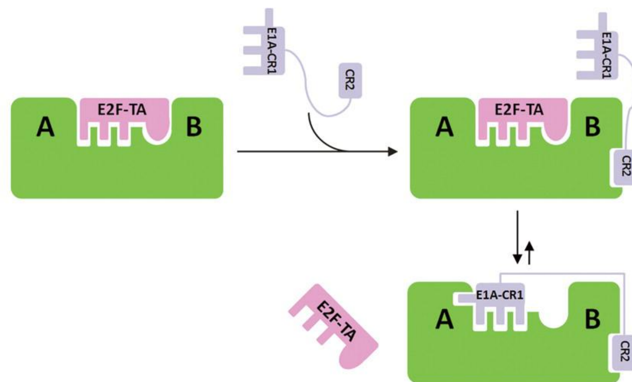

Figure 3: Schematic model for E2F-TA (transactivation domain) displacement from pRB by E1A. E1A binds with its CR2 domain to the LxCxE binding domain of pRB. A long flexible linker connects CR2 and CR1, which can now displace E2F and tightly bind to pRB. From (Liu and Marmorstein 2007).

Also the C-terminal domain is required for full growth suppression and maximal repression of E2F-responsive promoters. The binding of only a fragment of E2F alone to pRB is very likely a situation that does not occur in vivo. As mentioned earlier E2F forms a heterodimer and binds to the DP-class of transcription factors. The crystal structure of the C-terminal region of pRB (RbC) together with core domains of E2F1 and DP1 revealed an intertwined heterodimer in which both E2F1 and DP1 contact RbC (Rubin et al. 2005) (A schematic model is shown in Figure 4). This study provided biochemical data that a core fragment of E2F could bind the carboxy-terminus of pRB only when dimerized to a fragment of DP1. Phosphorylation of the C-terminus of pRB leads to elimination of the interaction of RbC and the E2F1/DP1 dimer providing a first mechanistic description of how the pRB-E2F-DP1 complex is dissociated by phosphorylation (Rubin et al. 2005).

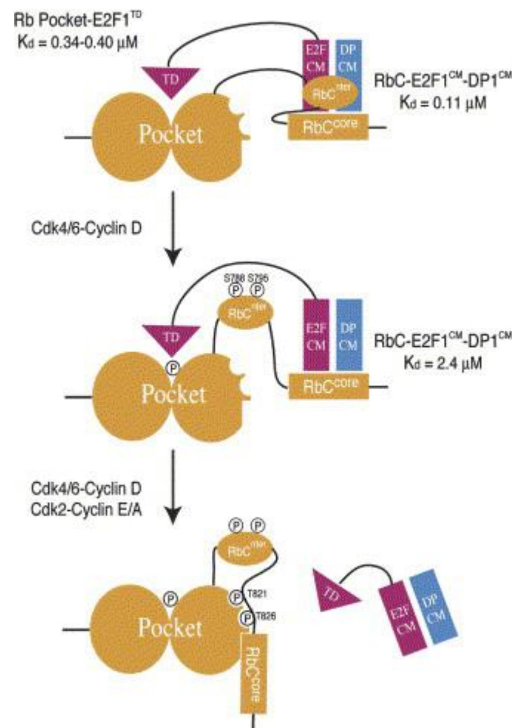

Figure 4: Model for phosphorylation induced dissociation of the pRB/E2F/DP complex. The Rb pocket binds to the E2F transactivating domain (TD), the C-terminus of pRB (subdivided into RbC-N-terminus and RbC-core) bind to the E2F/DP heterodimer (CM stands for coiled-coil plus marked-box domain, which mediate the interaction of E2F/DP and pRB). In early G1, phosphorylation of RbC-core results in reduction of overall RbC-E2F/DP affinity. Subsequent phosphorylation of the C-terminus of pRB induces intramolecular interaction between RbC and the Rb pocket, which ultimately results in disruption of the pRB/E2F/DP complex. From (Rubin et al. 2005).

Finally after elucidation of structures for the A/B pocket and the carboxy-terminus, a recent crystal structure of the N-terminus of pRB (RbN) completed our knowledge of the shape of RBR. Interestingly the structure of RbN consists of tandem cyclin like folds with a spacer between them, which is reminiscent of the architecture of the pRB A/B pocket (Hassler et al. 2007). Structure-guided sequence alignment revealed an above-average identity between RbN and the Rb pocket. This indicates that these two parts of pRB are homologs and suggests that Rb probably arose through duplication of an ancestral cyclin fold pair (Hassler et al. 2007). Although highly conserved regions occur on the N-terminus its importance for full tumor suppression has been unclear for a long time. For example Riley et al (Riley et al. 1997) could neither complement the embryonic lethal RB1-/- nor the tumor prone phenotype of RB-/+ mice with a version of RB that carried a small in-frame mutation in the amino-terminus (Riley et al. 1997). In contrast to that, Yang et al found that RB null embryonic stem cells reconstituted with a completely N-terminal truncated RB could rescue the tumor prone phenotype of chimeric animals

(Yang et al. 2002). The new finding that the disruption of the structure of RbN in vivo impacts the integrity of the whole protein resolves this conundrum whereby a mutation in RbN results in greater loss than total ablation (Hassler et al. 2007). Their results further indicate that the interaction of RbN and the pocket domain is not static but very dynamic with a closed and an open confirmation and the switch between those is modulated by other pRB binding proteins.

All these structural studies of pRB provide valuable insights into the mechanistic interaction with other proteins and surprisingly also on the evolution of pRB. The structural integrity of the whole pRB protein can explain the fact that although there are at least 35 known cancer causing missense mutations, none of them offers a discrete separation of activities of pRB (Dick 2007). This has to be taken into consideration for planning experiments that aim for dissection of the functions of retinoblastoma-related proteins.

More than 100 interaction partners of RBR have been reported (Morris and Dyson 2001) and an increasing number is found. More structural information is required to understand how pRB can engage in all these interactions, how these complexes are assembled and potentially bind to specific DNA-sequences and how binding affinities are modified by various posttranscriptional modifications.

Retinoblastoma-related proteins in cell cycle and development

Development of multicellular organisms relies on different programs to produce shape and cell specificity: differential cell division, differential cell growth, apoptosis and cell differentiation. The coordination of these events is achieved by intercellular, intracellular and especially for plants – external environmental signals. Whichever of these paths a single cell in a developing organism follows, it always has to pass through the cell cycle and decide whether to commit itself to another cycle, transiently exit cell cycle, or stop cycling at all. A protein that can stop the cell cycle may thus be an excellent target for extracellular signals to coordinate cell division in a developing organ. RBR has been of increasing interest for developmental biologist because it seems to be a likely candidate for an integrator of external information and internal cell signals.

Tales from unicellular organisms

Retinoblastoma-related genes have so far been found only in two heterotrophic unicellular organisms. In the ciliate *Eufolliculina uhligi* and in *Dictyostelium discoideum*. Interestingly, both organisms are able to produce different cell types with different functions and are thus developmentally special amongst their relatives.

Ciliates are probably the most complex patterned unicellular organisms. *Eufolliculina uhligi* belongs to the more basal group of heterotrich ciliates. *E. uhligi* has a distinct life cycle; in the motile stage a swarmer searches for a suitable place to settle where it undergoes metamorphosis to become a sessile, so-called trophont (Mulisch and Patterson 1987). Since the swarmer is not able to feed, grow, regenerate or reproduce, its cell cycle appears to be arrested. The trophont feeds, grows and reproduces by asymmetric cell division. Markmann-Mulisch isolated trophont-specific cDNA clones which contained, amongst other cell cycle regulating genes, a homolog to *pRB* (Markmann-Mulisch et al. 1999). They could furthermore confirm the trophont specific expression of this gene by Northern-blot analysis. Unfortunately this work has not been followed up so we can only speculate about the function of RBR in this organism. However, the specific expression in the trophont suggests that RBR in *E. uhligi* might be important for coordination of the cell cycle.

A little more is known about the function of *rblaA*, the ortholog of pRB in the social amoeba *Dictyostelium discoideum*. *Dictyostelium* cells aggregate when nutrition is exhausted and after a short multicellular migration phase, most of the amoebae differentiate as spores, dehydrated and encapsulated cells that can survive for long periods under hostile conditions. A minority of cells differentiates to become stalk cells, which elevate the spore mass from the substrate and facilitate dispersal. *rblA* expression correlated with the onset of cell-type specific gene expression and a *rblA::*α*pgal* reporter demonstrated the specificity of *rblA* for the spore pathway (MacWilliams et al. 2006). In accordance with that, when *rblA*- cells were mixed with wild type cells, *rblA*- cells showed a strong bias for the stalk fate. Furthermore in *rblA*- cells the length of the cell cycle did not seem be altered but *rbA*- cells had approximately only 50% of the average wild type volume. *rblA* also seemed to be not important for development in *Dictyostelium*; *rblA*-null cells showed accelerated development into fruiting bodies and formed patterns suggestive of aggregation streams on rich medium on which wild type cells never initiate development. Cells over-expressing *rblA* grew slow and showed increased duration of G1 (MacWilliams et al. 2006). Thus it seems that in *Dictyostelium*, *rblA* seems not to be important for cell differentiation but rather for the regulation of cell size and timing of initiation of development of the fruiting body.

C.elegans pRB promotes cell fate

C.elegans contains all members of the canonical pRB pathway; Lineage-abnormal-35, *lin-35* is similar to pRB, *dpl-1* similar to DP and *efl-1* similar to E2F (Lu and Horvitz 1998; Ceol and Horvitz 2001). The *lin-35* single mutant has no reported cell-cycle phenotype but can enhance the phenotype of other cell cycle mutants (Saito et al. 2004; Grishok and Sharp 2005; Ouellet and Roy 2007). However *lin-35* can rescue the cell cycle arrest in *cyd-1* or *cdk-4*, *C. elegans* homologs of the human cyclin D and CDK4/6 respectively – which supports the idea that *lin-35* has at least redundant effects on cell cycle progression.

The most striking consequences of Rb-pathway mutations in *C.elegans* are developmental defects. Double mutant worms, in which two genes of the RB pathway are mutated develop multiple vulva-like structures. To suppress this so-called multivulva phenotype, *lin-35* was not required in vulval precursor cells, but in the epidermis that flanks these cells (Myers and Greenwald 2005). From this study it has been inferred that *lin-35* is important to repress an epidermal growth factor, which induces the formation of vulva-like structures. This illustrated that *lin-35* is needed to restrict the expression of developmentally regulated genes to their proper context – which is the essence of many studies of the pRb-pathway in *C.elegans* (van den Heuvel and Dyson 2008). Interestingly *lin-35*, *efl-1* and *dpl-1* mutants show enhanced RNA interference and transgene silencing in the soma (Wang et al. 2005). The explanation for that is that these mutants allow chromatin remodeling factors, which are usually present only in the germline, to become active in the soma, which leads to transgene silencing, RNAi hypersensitivity and possibly most other features of the mutant phenotypes. Germlines need to maintain a totipotent state. In this example pRB-E2F might promote differentiation at least in part by counteracting transcriptional programs that are associated with the undifferentiated state (van den Heuvel and Dyson 2008). Taken together, studies in *C.elegans* demonstrated the importance of the retinoblastoma pathway not only in cell cycle but also for determining specific cell fates.

RBF1 and RBF2 in *Drosophila* development

Drosophila contains two retinoblastoma-like proteins, RBF1 and RBF2 (Du et al. 1996; Stevaux et al. 2002) and two E2F-like proteins – dE2f1 and dE2F2 with activating and repressing activity respectively (Dynlacht et al. 1994; Ohtani and Nevins 1994; Sawado et al. 1998). Homozygous *rbf1*-mutants die during larval stages and show deregulated expression of E2F-target genes, ectopic S-phases and elevated apoptosis (Du and Dyson 1999; Moon et al. 2006). Overexpression of dE2F1 induces ectopic S phases and apoptosis and can be suppressed by RBF1 (Du et al. 1996; Duronio et al. 1996). The effects of overexpressing E2F1 are especially conspicuous in transiently quiescent cells that have not yet committed to a differentiated state, such as cells in the imaginal discs (van den Heuvel and Dyson 2008). Gene expression profiling of *Drosophila* mutant cells showed that the dE2F-dDP-RBF pathway regulates a broad range of genes (Cayirlioglu et al. 2003; Dimova et al. 2003; Stevaux et al. 2005). These studies showed that not only classical E2F target genes such as S-phase genes were repressed via E2F/RB but also differentiation factors that are not cell cycle but developmentally regulated (Dimova et al. 2003). Moreover, two developmentally important pathways, wingless and Notch signaling can modulate dE2F1 and RBF1 activities (Duman-Scheel et al. 2004; Baonza and Freeman 2005), providing a good example of how the cell cycle can be coupled to patterning events. Taken together, RB/E2F complexes in *Drosophila* control not exclusively the G1-S-phase transistion but are also important mediators of development and cell cycle.

pRBs are tumor suppressors in mammalian cells

Most studies of pRB have been performed in mammalian systems which is not surprising giving its importance in tumor suppression. Mammals contain three pRB homologs, pRB or p105, p107 and p130 that act in the same pathway and have some degree of redundancy. All three are structurally similar and can bind to E2Fs (E2F1-5) and serve as substrates for cyclin dependent kinases (Cobrinik 2005). Although there might be some preference of different pocket proteins for different E2Fs (Hurford et al. 1997) especially p107 and p130 are to a large extent redundant (Wells et al. 2000). Developmental defects can be enhanced by mutations in p107 or 130 but only pRB is required for embryonic development and tumor suppression. *Rb* null mice die 14-15 days after gestation and exhibit neuronal cell death and defects in erythropoesis (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). Heterozygous *Rb* null mice as well as humans develop normally but spontaneously develop a variety of different tumors ((Bookstein and Lee 1991; Hu et al. 1994; Zhou et al. 2005). In contrast, mice completely lacking p107 or p130 develop normal, are not tumor prone and mutations of p107 or p130 is rarely observed in human cancer (Goodrich 2006). This tumor suppressor function of pRB was originally thought to be mainly due to its function as a checkpoint control at

G1/S-phase transition via inhibition of E2F transcription factors. In order to pass that checkpoint CDK4/6-cyclin D and CDK2-cyclinE have to be activated. These can then phophorylate pRB which loses its affinity to E2F transcription factors. E2Fs then can activate the gene-set necessary for S-phase entry. The repressive function of pRB can be reinforced by the interaction with chromatin-modifiers. The histone methyltransferase DNMT1 can form a stable complex with pRb, E2F1 and HDAC1. Loss of functional pRb may result in inappropriate activity of the complex and may account for both hypomethylation and local hypermethylation often observed in tumor cells (Robertson et al. 2000).

The Rb pathway may also be involved in G2/M-phase control. Many M-phase regulatory genes such as *Cdk1*, *CycB*, *Plk1*, and *Cdc20* were found to be regulated by RB/E2F in microarray experiments (Ishida et al. 2001). The mitotic checkpoint protein Mad2 is a direct target of E2F and is aberrantly expressed in cells with pRB pathway defects. Misexpression of Mad2 predisposes cells to genomic instability which leads to aneuploidy (Hernando et al. 2004) and formation of highly aggressive cancer in mice (Sotillo et al. 2007). Additionally pRB localizes to the mitotic spindle and can bind beta-tubulin (Thomas et al. 1996), thus demonstrating a possible mechanical role of pRB during mitosis. RBR can also physically interact with components of the anaphase promoting complex (APC) and this interaction mediates the ubiquitin dependent degradation of the F-box protein Skp2 of the ubiquitin ligase Scf-Skp2. Since Scf-Skp2 targets kinase inhibitor p27kip1 for ubiquitination, interaction of pRB and APC results in a stabilization of p27kip1 (Amati and Vlach 1999; Binne et al. 2007). This results in a stabilization of pRB because p27kip1 is an inhibitor of cyclin/CDK complexes.

Finally pRB seems to have an important role in promoting cell differentiation, which might contribute to its tumor-suppressing activity. *Rb* null mice show many differentiation defects in specific cell types. Mechanistically this is achieved by binding and regulating tissue-specific transcription factors and inhibitors of differentiation. For example pRB is required for induction of certain muscle-specific genes via the interaction with MyoD (Skapek et al. 2006). This is achieved by direct physical inhibition of the E1A-like inhibitor of differentiation Eid-1, which represses the histone-acteyl-transferase p300, an essential coactivator of MyoD (MacLellan et al. 2000). Furthermore pRB can act as a molecular switch determining white vs. brown adipogenesis via regulating the expression of the brown fat-specific uncoupling protein 1 (Ucp-1) (Hansen et al. 2004).pRB has also been shown to be important for erythropoesis; Rb promotes differentiation of macrophages by opposing the inhibitory functions of Id2 on the master regulator for macrophage differentiation, Pu.1 (Iavarone et al. 2004).

Studies of the tumor-repressive functions of pRB in mammalian cells revealed three major types of actions: - direct inhibition of cell cycle progression via the E2F/DP/CDK and CDK inhibitor machinery, modification of transcription via chromatin-modifiers and

promotion of differentiation by interacting with proteins necessary for cell fate decisions.

Retinoblastoma-like proteins in plants

Until proven wrong by Shen et al 1994 (Shen et al. 1994) retinoblastoma-related proteins (RBRs) have been thought to occur only in the animal lineage (Durfee et al. 2000). In fact, it took more than 12 years for this information to arrive in the animal field as impressively demonstrated by the title of a review published 2006 in Oncogene: "Rb, whi its not just for metazoans anymore" (Cooper 2006). (Whi referring to the newly discovered yeast-analog of RB).

However, the occurrence of retinoblastoma related proteins in plants and animals shows that RBRs must have been present in a common unicellular ancestor. That strongly suggested that specific functions of RBR in differentiation and development are more derived features and that its primal role was regulation of the cell cycle.

Most of the cell cycle regulating genes are conserved between plants and animals but many developmental processes are very different. First, many apparently fully differentiated plant cells are totipotent; due to the cell wall, plant cells cannot move; plants have an alteration of generations, plants show postembryonic development and unlike animals, plants do not set aside a pool of cells for the germ-line but produce meiocytes from continuously proliferating meristems. The conserved players of the cell cycle on one side and very different developmental processes as animals on the other made it attractive to study the function of RBR in plants as new model-organisms.

Function of retinoblastoma related protein in *Chlamydomonas* and *Volvox*

Chlamydomonas reinhardtii is a unicellular green alga and contains a single-copy pRB homolog which is called Mat3. *mat3* was identified in a screen of insertional mutants displaying a small size phenotype (Umen and Goodenough 2001). *Chlamydomonas* has a fission cell cycle; in the long G1 phase, cells can increase in volume several times. After passing a restriction point in early/mid G1 cells will complete the cell cycle, even if subsequent growth is stopped by withdrawal of light or nutrients (Umen and Goodenough 2001). In order to pass commitment, a minimum cell size has to be attained. After commitment cells continue to grow and finally undergo a rapid series of alternating S phases and mitoses to produce several daughter cells. The mother cell size controls the number of S/M and thus daughter cell size can be used as a direct readout of cell size checkpoint function (Umen 2005). *mat3* mutants have the length of G1 but

pass the commitment point at a premature cell size and undergo more rounds of S/M cycles than wild type. This results in daughter cells 25-35% smaller than wild type. In a screen looking for suppressors of the *mat3* phenotype homologs of E2F and DP were identified. Interestingly *dp1* null mutants had a large-cell phenotype that could only slightly be rescued by *mat3*. Suppression of *mat3* by *dp1* and *e2f1* was not caused by a prolonged cell cycle but due to a defect in size checkpoint control. Thus Mat3/RB is a negative regulator of cell cycle progression in Chlamydomonas and seems to control also the cell size checkpoint (Umen and Goodenough 2001).

A very interesting study that sheds some light on the path a cell-cycle regulator might follow during the evolution of multi-cellularity is about the retinoblastoma-related protein from the green alga *Volvox carteri.* Consisting of only two cell types – somatic and germ cells – this alga represents one of the simplest multicellular organisms. It is assumed that the multicellularity of *Volvox* evolved very recent from an unicellular *Chlamydomonas* like ancestor (Rausch et al. 1989). In *V.carteri* males and females exist and are able to both asexual and sexual reproduction. Sexual reproduction can be induced by a sexinducer glycoprotein which triggers development of large nonmotile female gametes and small motile male gametes in females and males respectively (Starr 1970; Starr and Jaenicke 1974; Tschochner et al. 1987). Surprisingly, only females contained a RBR gene which is localized close to the mating-type locus, but males, which display the same vegetative development as females do not contain RBR (Kianianmomeni et al. 2008). Males that were transformed with the female RBR showed several phenotypic differences to wild type. They displayed a reduced number of increased gonidia, an increased cell volume of somatic and gonidial cells, a prolonged embryo development, increased diameter of sperm packets and interestingly – concordantly to the pale *mat3*-mutant in *Chlamydomonas* an increased chlorophyll content (Kianianmomeni et al. 2008).

The increased cell size in male cells expressing female RBR is in accordance to the *Chlamydomonas mat3* mutant, which shows a decrease in cell size. What is somewhat puzzling is the absence of RBR in wild type males despite the identical vegetative development of males and females. Kianianmomeni discuss (Kianianmomeni et al. 2008) that either a functional analog exists in males or that RBR in males evolved so rapidly that it was not detectable by southernblotting or pcr with degenerated primer. This might be explainable by the fact, that sex-related genes at the mating type locus can evolve very fast (Lageix et al. 2007).

RBR in gametophyte development

In flowering plants a very precise, spatial and temporal regulated program takes place to integrate growth, patterning and differentiation in the highly derived, minimized male and female gametophytes. The female gametophyte originates from one haploid meiotic megaspore which undergoes three successive mitotic division to generate a syncytium with eight nuclei (Yadegari and Drews 2004). Cellularisation and differentiation results in a seven-cell structure consisting of three antipodals, two synergids, and two female gametes, the egg cell and the diploid central cell, which results from the fusion of two haploid polar nuclei (Yadegari and Drews 2004). Finally the three antipodals degenerate and the mature female gametophyte contains the egg cell, the central cell and two synergids. The female gametophyte is embedded within the diploid integuments and this whole structure is called ovule. Sexual reproduction takes place in the ovule and the mature female gametophyte awaits double fertilization. The fusion product of one sperm cell and the egg cell will give rise to the zygote and the second sperm cell fuses with the central cell to give rise to triploid endosperm.

Knockout alleles of RBR1, the only homolog of pRB in Arabidopsis, are gametophytic lethal. Therefore 50% of ovules do not develop seeds and abort when the mutant allele is inherited maternally (Ebel et al. 2004). The mature gametophyte fails to arrest mitosis and shows aberrant nuclear proliferation close to the micropylar end and the central cell (Ebel et al. 2004). The overproliferating nuclei of the central cell do not express an endosperm marker suggesting that they do not adopt endosperm but instead retain gametopyhtic identity (Ingouff et al. 2006). Thus in the *Arabidopsis* female gametophyte RBR seems to fulfill its classical role as a negative cell cycle regulator.

As for the female, also the male germ lineage forms after meiosis. Asymmetric division of haploid microspores results in one vegetative cell that encloses a germ cell which undergoes one further division to produce two sperm cells (McCormick 2004). *rbr* mutant pollen has severely reduced transmission efficiency of the paternal *rbr* allele (Ebel et al. 2004). In more than 40% of *rbr/RBR* pollen the vegetative-like nucleus had undergone an additional round of mitosis. This resulted in a failure to form sperm cells and aborted pollen with diffused chromatin. Sometimes also an aberrant number of sperm cells formed (Johnston et al. 2008). This suggests that RBR is required for proper differentiation of sperm cells or cell cycle control in the vegetative cell. Taken together RBR seems to affect mitotic events and cell specification in *Arabidopsis* male and female gametophytes.

RBR in sporophyte development

The retinoblastoma-pathway is conserved in all plants investigated so far from bryophytes to angiosperms. The genome of dicots contains only one RBR related protein whereas monocotyledonous species possess usually two types of RBR genes. In maize the two different RBR proteins RBR1 and RBR3 seem to have complementary functions. Both appear to be regulated by phosphorylation via CDKs. During endosperm development RBR3 is present only during the mitotic phase and the levels of RBR1 are high also later in development (Sabelli et al. 2005). This could suggest a division of labor whereby RBR3 participates in cell cycle control and RBR1 in cell differentiation (Inze and De Veylder 2006).

Many studies on *Arabidopsis* have established an important role of RBRs in the plant cell cycle (a simplified model is presented in Figure 5). Overexpression of the RBR interaction partner E2Fa induced sustained cell proliferation in seedlings. This phenotype was strongly enhanced by co-expressing the dimerization partner of E2Fa, DPa. E2Fa/DPa overexpressing plants arrested early in development, S-phase genes were strongly induced and endoreduplication was enhanced (De Veylder et al. 2002). Accordingly, reducing RBR levels in *Nicotiana benthamiana* with virus induced gene silencing (VIGS) resulted in prolonged cell proliferation and delayed cell differentiation in leaves and stems, induced expression of E2F regulated genes and increased endoreduplication (Park et al. 2005).

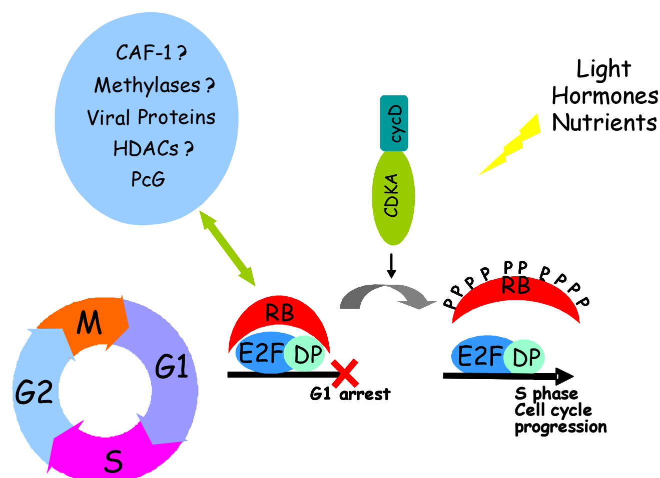

Figure 5: Simplified model for the role of RBR during the plant cell cycle. Similar to animal pRB, RBR seems to be an important player in regulating the G1/S transistion. Upon inactivation of RBR by CDK/cycD, E2F/DP transcription factors are released which results in expression of genes necessary for S-phase. The cell cycle machinery has to integrate external signals with developmental positional and epigenetic signals. As a consequence cells maintain proliferation competence, become quiescent, differentiate or die.

Similar results were obtained by Desvoyes et al. when altering the activity of RBR in *Arabidopsis* using an inducible version of the geminivirus RepA protein. RepA interacts with RBR through an LxCxE amino acid motif (Xie et al. 1995) regulates viral DNA replication and has been used before to address the role of RBR in proliferation of cultured tobacco cells (Gordon-Kamm et al. 2002). Inducible overexpression of *RepA* restricted cell division at early leaf development. Pavement cells underwent extra rounds of cell division. This resulted in an approximately four-fold size reduction but cells maintained their differentiation state. Trichomes and stomata showed not change in proliferation state or fate specification. Overall, leaves cells showed an increase in endoreduplication. From these results, the authors concluded that RBR inactivation has different effects on cell division and endoreduplication of different leaf cell types. The conditional expression of Clink, a nanovirus protein that also targets RBR in *Arabidopsis* resulted in very similar phenotypic alterations and in the same conclusions about the function of RBR (Lageix et al. 2007). In *Arabidopsis* roots, loss of RBR leads to expansion in the stem cell pool seemingly without affecting their self-renewal potential by preventing their differentiation (Wildwater et al. 2005). Wyrzykowska et al addressed this question at the shoot apical meristem of tobacco (Wyrzykowska et al. 2006). The authors were able to trigger cells towards a more differentiated state when inducing a local and transient overexpression of RB in the tobacco shoot apical meristem.

Although these studies paved the way for finding the functions of RBR in plants most of the effects found from deregulating RBR could be simply explained by altering the duration of the proliferating state of various cell types. Indirect evidence that RBR could be involved in more global cell fate decisions and may regulate more specific differentiation events in plants comes from the study of RBR interacting partners; FVE was physically associated with RBR in immunoprecipitation assays. The *fve* mutant is late flowering, i.e. the change from vegetative growth to reproductive growth is delayed. This correlates with an increased histone-acetylation of the negative flowering control-factor FLC (Ausin et al. 2004). FVE belongs to one of five WD40 MSI-like proteins from *Arabidopsis* (Hennig et al. 2005). Msi1 is – similar to RBR – also involved in gametophyte and seed development (Kohler et al. 2003). *Arabidopsis* plants co-suppressed for *MSI1* also showed a late flowering phenotype and gene expression correlated strongly with *fve* (Bouveret et al. 2006). Futhermore MSI1 as member of the polycombgroup repressive complex was able to influence the chromatin state of another flowering time regulator, AGL19 showing a direct link to formation of repressive H3K27 histone methylation (Schonrock et al. 2006). However, the involvement of RBR in these processes remains to be determined.

Taken together studies from plants strongly suggest that the RBR/E2F/DP pathway is - similar to the situation in animals - a crucial regulator of the plant cell cycle. However relatively little is known about how this pathway connects external signals, like light,

sucrose and hormones to developmental, positional and epigenetic signals and how this can modulate cell fate decisions.

2. Goals of this study

The objective of this work was to carry out a functional investigation of the retinoblastoma- related protein. Research from all kinds of model organisms has established that RBR is a negative regulator of the cell cycle. Animal research was able to provide many examples where pRB has a direct link to cell fate decision thus linking cell cycle control to differentiation. Still, even in animal systems and despite two decades of intensive research, many aspects of pRB are not understood. For example, are these effects on differentiation in certain cell types only exceptions or is there a more general effect of pRB on cell fate? And may that be connected to the question why pRB is such an important target in developing cancers?

Compared to the work about pRB that has been done especially in mammalian cells, the study of its function in plants is only in its infancy. Because most plant organs develop post-embryonically they offer an excellent system to address questions concerning whether cell division is the driver of growth and development or if cell division merely follows a developmental plan. Also plants undergo several developmental switches, in which cells of certain regions change their identity; For example the whole embryo switches from an embryo morphogenetic growth to a maturation phase and after maturation and germination from heterotrophic growth to an autotrophic seedling growth. Later the shoot apical meristem switches from a vegetative leaf producing growth to a reproductive flower producing growth. Another striking feature of plants is that they are remarkably resistant to neoplastic transformation and plant tumors develop only after infection with specialized pathogens such as *Agrobacterium*.

pRB as an important cell cycle regulator is an attractive candidate that could be involved in all these processes. This is why we chose a bottom up approach and studied the plant retinoblastoma-related protein and its role in the context of these processes. Because plants and animals diverged from a common unicellular ancestor we would expect that also in plants pRB is an important player in the cell cycle. And recent publications on RBR in plants gave first evidence that this is indeed the case. However only asymmetric cells or multicellular organisms show differentiation and development. That means it would be very interesting to see if RBR is important for cell fate decisions and development in plants as well. And information about these topics could shed light on general concepts of development and neoplastic transformation. Finally for establishing RBR as field of research in developmental plant biology it will certainly be important to define the interactions of RBR with other proteins and understand the regulation of its expression.

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3. *Arabidopsis* retinoblastoma related protein is necessary for embryo to seedling transition

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Abstract

Previous studies have established that similar to the situation in animals, plant retinoblastoma-related (RBR) proteins are crucial regulators of the cell cycle. However, the role of RBRs in differentiation, cell fate determination and thus plant development remains controversial.

During sporophytic development, plants undergo several phase transitions. One of them is the transition from a heterotrophic embryo to an autotrophic seedling. In the seed maturation phase embryos import nutrients and store reserves that are degraded and mobilized to support this transition after germination. Several genes involved in this process have been identified, for example genes coding for the family of B3 domain transcription factors. Yet none of them has been linked to cell cycle control.

Here we show that reducing the levels of RBR during embryo development leads to a block of post-germination seedling development. When grown on sucrose, RBR deficient mutants developed swollen hypocotyls and cotyledons due to massive ectopic cell proliferation. Notably, embryonic marker and seed maturation genes that were not sugar-inducible in wild-type seedlings were strongly reactivated in *Arabidopsis* mutants with reduced RBR levels. Amongst those were two master regulators of embryonic development, *LEC2* and *ABI3*.

Taken together, our results indicate that reducing RBR in *Arabidopsis* hypersensitizes cells to sucrose stimulation and that RBR is part of a regulatory circuit repressing the seed maturation program after germination.

Introduction

In animals, retinoblastoma-related proteins have been shown to be key regulators controlling the entry into the cell cycle (Lee et al. 1987; Goodrich et al. 1991; Ebel et al. 2004). Interactions 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. Phosphorylation by CDKE/D releases the RB-mediated inhibition and allows cell cycle progression (reviewed by (Burkhart and Sage 2008; van den Heuvel and Dyson 2008). Additionally to this role in cell cycle restriction and thus tumor suppression, retinoblastoma proteins have been directly linked to differentiation of certain cell types, e.g. of muscle cells, neurons, adipocytes and osteocytes (Gu et al. 1993; Martelli et al. 1994; Chen et al. 1996; Li et al. 1996; Thomas et al. 2001; Hansen et al. 2004), establishing pRBs as an important link of cell cycle progression and development.

In plants, pRB homologs have been discovered first in maize and later also in more basal members of the plant lineage such as *Chlamydomonas*, *Ostreococcus* and *Physcomitrella*. The *Chlamydomonas* pRB version seems to be important for cell size control via regulating E2F/DP homologs (Umen and Goodenough 2001; Fang et al. 2006).

The *Arabidopsis* genome contains one pRB homolog, the retinoblastoma related protein (RBR), which is necessary to arrest mitosis in the embryo sac (Ebel et al. 2004). Thus, knockout alleles of RBR are gametophytic lethal, which makes it difficult to study the role of RBR for cell cycle, differentiation and development in the sporophyte. To overcome this problem, virus induced gene silencing (VIGS) of RBR in tobacco and viral proteins to target RBR in *Arabidopsis* have been successfully used (Park et al. 2005; Desvoyes et al. 2006; Lageix et al. 2007). These studies demonstrated the importance of *RBR* for cell proliferation, differentiation and endoreduplication in leaf and stem tissue late in plant development. Similar phenotypes were obtained in plants that overexpressed simultaneously E2Fa and DPa transcription factors (De Veylder et al. 2002). Other studies more focused on meristematic cell maintainence, could demonstrate that a local, transient overexpression of RBR protein in the shoot apical meristem of tobacco triggered premature differentiation (Wyrzykowska et al. 2006), and that a local reduction of RBR in the root apical meristem lead to an increased number of stem cells (Wildwater et al. 2005). How RBR mediates the interaction of cell proliferation and differentiation remains an open question.

Given the prominent role of RBR in cell cycle regulation, one might also expect RBR to be important earlier in sporophyte development. However, the question of the role of RBR during embryogenesis and seedling establishment has not been addressed so far.

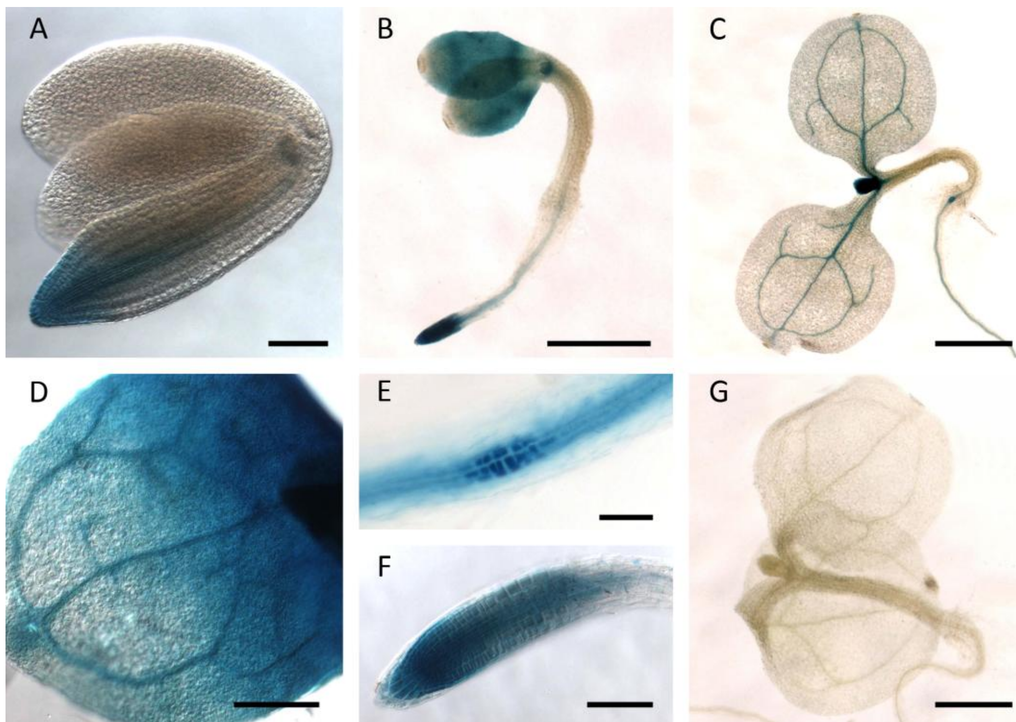
Embryo development in seed plants consists of two phases: morphogenesis and maturation. In the maturation phase synthesis and accumulation of storage macromolecules and acquisition of desiccation tolerance occurs (Braybrook and Harada 2008). Seed maturation depends on the import of sugar and other nutrients and during seedling development the seed reserves are rapidly degraded (Tsukagoshi et al. 2007). Known mutants that affect these processes, for example *abi5* and *abi4*, are often resistant to high concentrations of sugar and abscisic acid, which inhibit the germination of wild type seeds (Rolland et al. 2006). Other crucial regulators of late seed development belong to B3 domain transcriptional repressors, which comprise *LEC2*, *FUS3*, *HSI2*, *HSL1*, *HSL2* and *ABI3*. Overexpression of *ABI3* in seedlings lead to high expression of seed storage proteins and mutants strongly affected accumulation of seed storage proteins (Nakashima et al. 2006). *Hsi2* and *Hsl1* have recently been identified to prevent 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). Some studies suggested that metabolites such as sugars act as important signals regulating seed development and germination (Rolland et al. 2002; Weber et al. 2005). Hexokinase1 plays an important role in transmitting sugar signals into gene expression changes of this transcriptional regulators, but there might also be hexokinase1 independent pathways (Cho et al. 2006). Many of these transcription factors also seem to play a role in abscisic and gibberellic acid signaling, but relatively little is known how the switch from seed maturation to seedling growth is connected to cell cycle.

In this study we were able to generate *Arabidopsis* lines that had reduced levels of RBR early in sporophyte development. In accordance to previous work, mutant seedlings showed phenotypes that were related to defects in cell cycle regulation but also showed significant differences to other *Arabidopsis* systems with induced cell cycle activity (De Veylder et al. 2002; Park et al. 2005; Desvoyes et al. 2006; Lageix et al. 2007). Also we could show that mutant seedlings with reduced levels of RBR reacted hypersensitive to sugar stimulation, which resulted in ectopic, callus-like cell proliferation. This demonstrated the importance of RBR not only for cell cycle termination, but also for differentiation in the context of a developing plant. Finally we provide evidence, that RBR is an important regulator of the embryo to seedling growth transition and likely acts via repressing sugar-inducible expression of embryonic genes. Thus, RBR is a novel integrator of sugar sensing and directly links embryonic to seedling phase transition to cell cycle control.

Results

RBR is mainly expressed in proliferating cells

To get insights into the potential role of *RBR* in sporophyte development after germination, we first investigated its transcriptional regulation. A genevestigator generated digital northern blot (Zimmermann et al. 2004) (Supplementary figure 1) showed that RBR is uniformly expressed across different tissues and different developmental stages. In order to reveal *RBR* expression pattern in more detail we transformed Col-0 plants with a *GUS*-reporter cloned downstream of the 5` promoter-region of *RBR.* Four independent transformed lines showed the same pattern of GUS-staining. One day after germination, GUS-staining was restricted to the root and the shoot apical meristems (Figure 1A). Two days after germination, additional *GUS*-expression was present in the cotyledons with exception of the hydatodes (Figure 1B). Three days after germination, GUS staining became restricted to the vasculature (Figure 1C). In older seedlings strong staining was always observed at the region of the shoot apex, in the vasculature, in young leaves (Figure 1D), in root tips (Figure 1F) and in emerging lateral roots (Figure 1E), suggesting a strong transcriptional activity of the *RBR* promoter in tissue with high cell division activity. This was especially evident in young leaves with strong GUS-staining in the proximal half of the leaf, where most cell division occurs (Figure 1D). In order to correlate *RBR*-promoter activity with RBR protein levels, we performed immunoblot analysis with affinity-purified antibodies against the N-terminus of RBR (specificity of the antibodies is shown in supplementary figure 2). Consistent with the *GUS*-reporter results, RBR protein levels were highest in young seedlings (3d old vs. 14d old), in roots, in the shoot apex and in young leaves (Figure 1H).



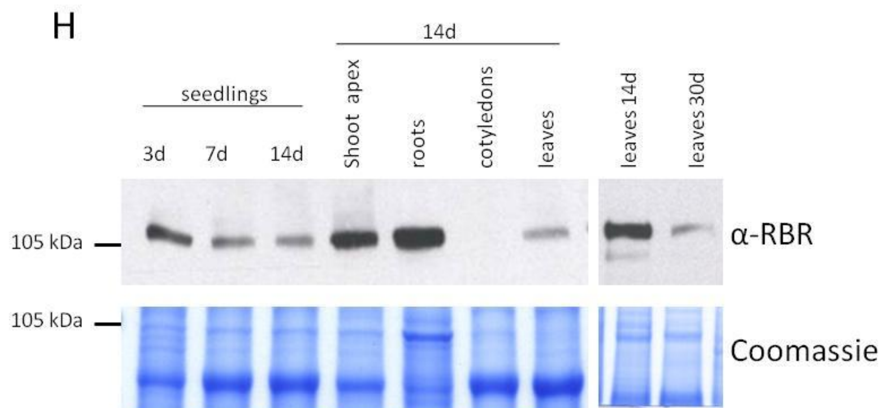


Figure 1. *RBR* is mainly expressed in proliferating tissue. *RBR* promoter activity was monitored in *RBRpromoter:GUS* seedlings. GUS staining had a conspicuous maximum in cotyledons two days after germination (A=1d.a.g, B=2d.a.g., C=3d.a.g). Also GUS staining was strong in dividing cells, such as root tips (F) in emerging lateral roots (E), the region around the root and shoot apical meristem and the proximal half of young leaves (C-F). As a control, a wild type plant is displayed in G.

H: Analysis of RBR protein. Proteins were analyzed by SDS-PAGE and immunoblotted with an antibody raised against the N-terminal domain of RBR (specificity of the antibody is shown in supplementary Figure S2). The migration of proteins of standard sizes (kDa) is indicated. A coomassie-gel with the same protein loading is shown as control. Individual tissues were collected from 14 or 30 day old seedlings grown under long day conditions. The scale bar in A, D, E and F indicates 100µm; in B and C 500µm.

Generation of plants with either ectopic or reduced RBR levels

Knockout alleles of RBR are gametophtic lethal and homozygous mutant plants cannot be obtained to study the function of *RBR* in sporophyte development (Ebel et al. 2004). Surprisingly, strong activity of a transgene may result in co-suppression thus leading to a reduced expression of the transgene and the endogenous gene (Matzke and Matzke 1995). Therefore we cloned the coding sequence of RBR under control of the viral 35S promoter and used a transformation vector that allows for visible selection of transformed T1 seeds (Stuitje et al. 2003) (Figure 2A). Almost all of the selected seeds germinated normally, but subsequently ceased growth and failed to develop leaves. Figure 2B shows that these seedlings had strongly reduced levels of RBR compared to the wild type. This decrease in RBR protein was likely the result of co-suppression hence these lines were named 35s*RBRcs*.

In a second approach, we cloned the coding sequence of *RBR* under control of the native *RBR* promoter (Figure 2A). This construct was originally produced to complement the *rbr/-* mutants. However this was not possible, indicating the existence of regulatory sequences on the genomic region. When we transformed Col-0 plants with this construct, T1 plants showed no discernable phenotypic abnormality. Among the progeny of 10 out of 23 independent lines up to 40% of seedlings arrested developmentally after germination and looked identical to the 35s*RBRcs* plantlets (Supplementary table 1). Western blot analysis of these seedlings confirmed a strong reduction of RBR protein levels in the arrested seedlings and showed an increase of RBR protein in normally developing seedlings (Figure 2B), demonstrating that a moderate increase in RBR protein had no adverse effect on *Arabidopsis* development. We concluded that the arrested seedlings were also co-suppressed for RBR and named these lines *RBRcs*, the wild type like looking sibling plants were named *RBR-OE*. In a third approach a RNA hairpin directed against the 5` end of *RBR* for constitutive silencing of *RBR* was constructed. This hairpin was under control of a 35S promoter and allowed for visible selection of transformed T0 seeds. Plants that germinated from these seeds showed the same phenotypic abnormalities as the *RBRcs* seedlings and a strong downregulation of RBR protein (Figure 2). These seedlings were named *35sRBRi* mutants. Intriguingly we found increased levels of *RBR* mRNA in all three systems, implying a negative correlation of *RBR* transcript and protein level (data not shown). Therefore only RBR protein measurement gave us a reliable indication of RBR activity in these mutants.

Taken together we concluded that the strong morphological changes in 35s*RBRcs*, *RBRcs* and *35sRBRi* were due to reduction of RBR levels.

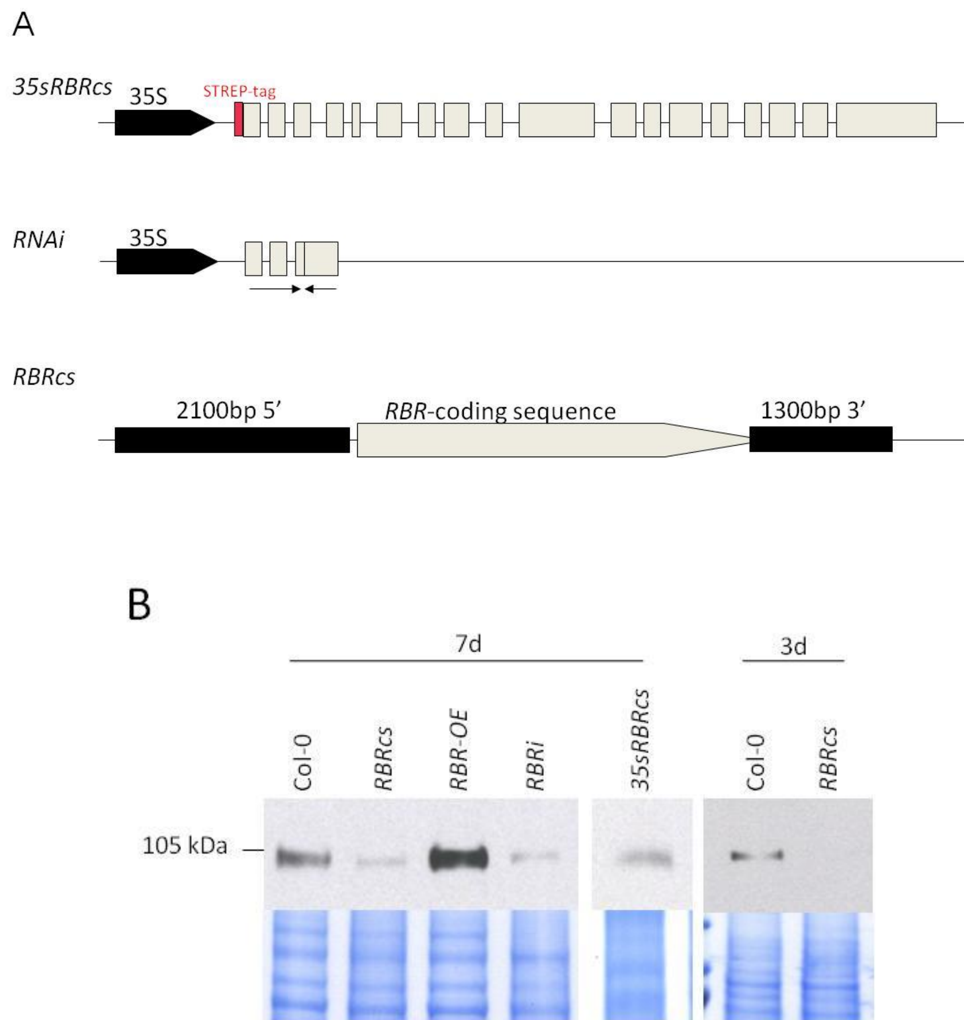


Figure 2.

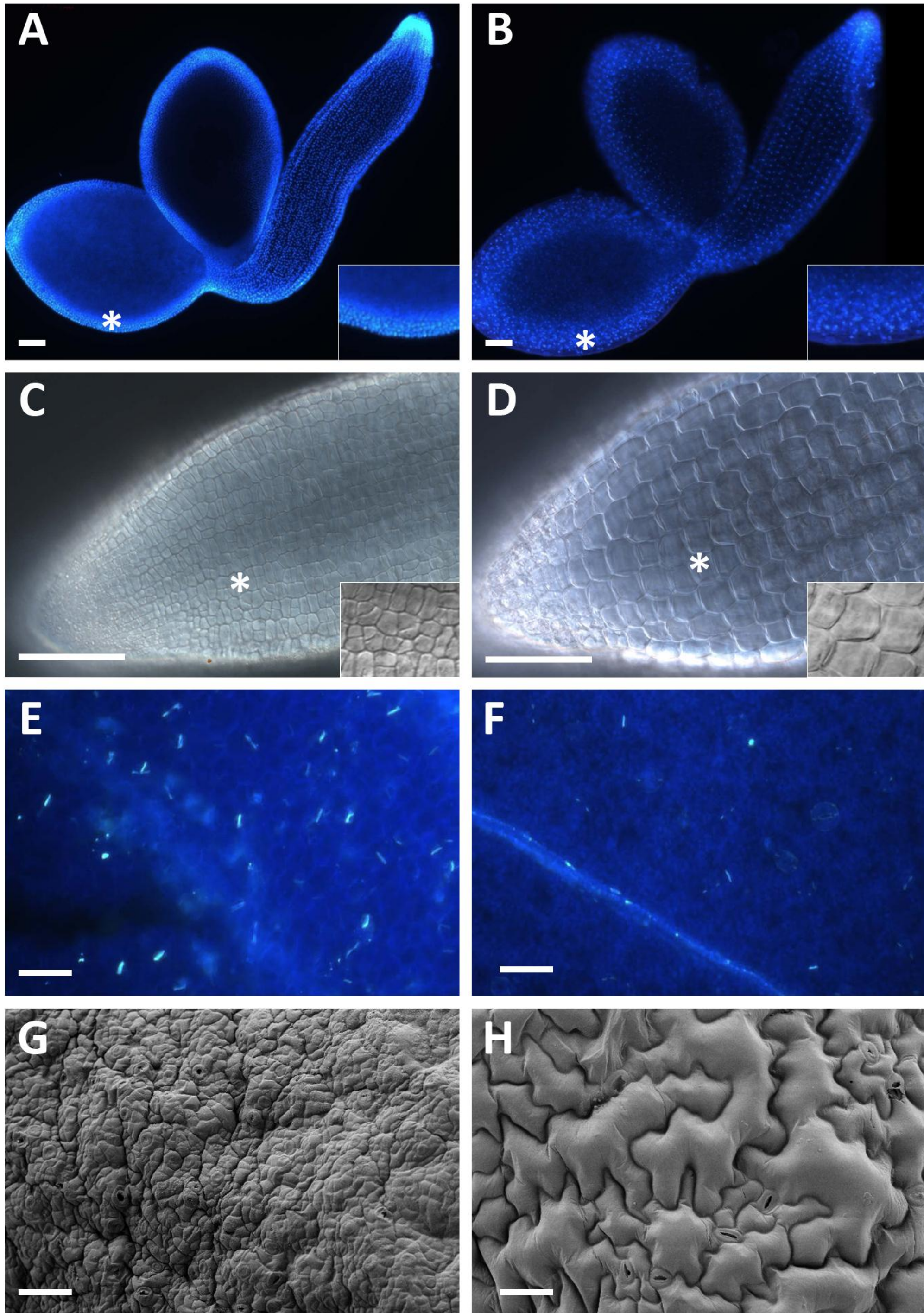
Generation of seedlings with reduced levels of RBR. A: simplified scheme of constructs used to downregulate RBR protein. The strong viral 35S promoter was either driving the expression of the genomic *RBR* region or a hairpin. The coding sequence with native up and down stream sequences gave rise to co-suppression *RBR* lines. Shown is also a strep-tag that was fused to the 5`end of *RBR*.

B: Protein levels of RBR in different lines. Western-blot analysis demonstrated a strong reduction of RBR protein levels in *RBRcs, 35sRBRi* and *35sRBRcs* lines compared to wild type at different time-points after germination. *RBR-OE* co-segregate with *RBRcs* mutants but were phenotypically indistinguishable from Col-0 seedlings.

Reducing RBR arrests seedling development

Seedlings with reduced RBR protein levels showed severe developmental defects. In order to reveal when the effects of RBR downregulation were established we dissected embryos of different growth stages and analyzed their morphology. All embryos of a line giving rise to *RBRcs* seedlings were morphological identical until the late bent cotelydon stage. At this stage we could distinguish embryos with an increased number of smaller cells from embryos with a normal appearance. The ratio of embryos with increased number of cells corresponded to the ratio of seedlings developing the *RBR* cosupression phenotype after germination. This showed that the reduction of RBR protein led to an increase in cell number in late embryogenesis. Along the hypocotyls of mature embryos we found 64+/-4 epidermal cells per cell file in *RBRcs* mutant embryos compared to 28+/-3 cells Col-0 in non-symptomatic embryos (Figure 3A, 3B and 3I, N=10; p=1.1E-13). Additionally there were more epidermal cell files on the hypocotyls of *RBRcs* embryos compared to wild type (Figure 3C and 3D plus insert) demonstrating not only ectopic anticlinal cell division but also increased periclinal cell division. DAPI-staining of nuclei revealed also an increased cell number of epidermal cells on the cotyledons of *RBRcs* seedlings (Figure 3A and 3B plus insert). Reducing RBR levels seemed not to affect the germination process since we could not detect differences in radical appearance. Three days after germination *RBRcs* mutants could be distinguished from normal looking siblings because they retained an apical hook, the cotyledons did not open and very often the hypocotyls displayed a strong curvature (Figure 4).

After an initial step of organ expansion *RBRcs* seedlings were developmentally arrested (Figure 4) and remained in this state for several weeks without visible further development or deterioration. The number of epidermal cells on the hypoctoyl of 7d old etiolated *RBRcs* seedlings was similar to that of mature embryos (Figure 3I) demonstrating that *RBRcs* seedlings, like wild type, grew by cell expansion and did not show additional ectopic cell divisions at this stage of development.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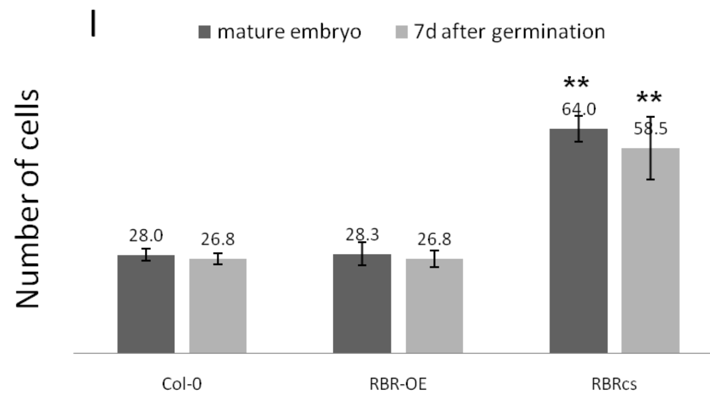


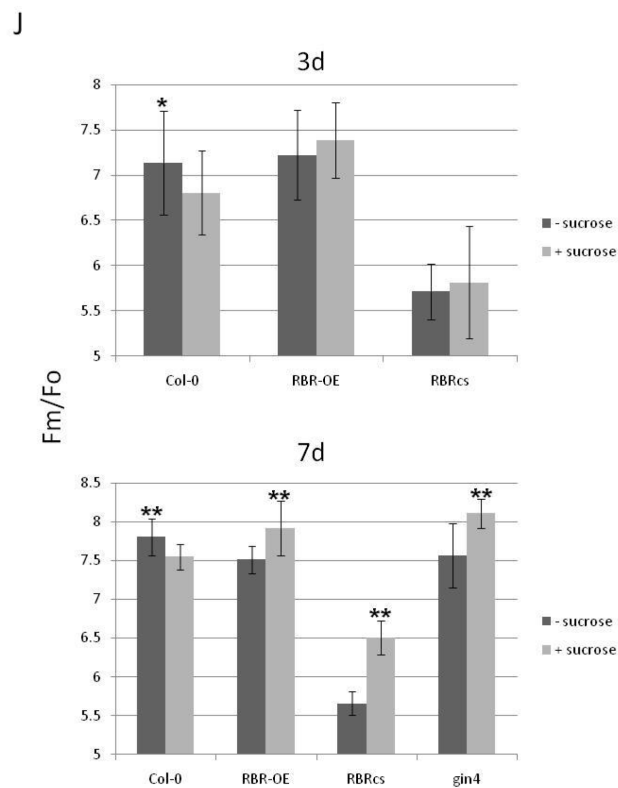


Figure 3.

Ectopic cell division in *RBRcs* mutants. Mature *RBRcs*-embryos (A) have an increased cell number compared to Col-0 embryos (B) as revealed by DAPI-stain of the nuclei. The inset shows the part of the cotyledon marked by an asteriks. The hypocotyls of *RBRcs* embryos (C) show not only an increased cell number along the main axis (number of cells along the hypocotyls is shown in I, ** indicates p<0.01 for the difference between *RBRcs* mutants and Col-0) but also an increased number of cell files compared to Col-0 embryos (D). The asteriks show the position of the insets.

E: Anilin-staining of phragmoplasts of 5d old *RBRcs* seedlings grown on medium supplemented with 1% sucrose shows increased cell division activity on cotyledons (E) compared to Col-0 seedlings (F).

G+H: SEM pictures of the surface of cotyledons of 14d old seedlings grown with sucrose showed massive ectopic cell division in *RBRcs* seedlings (G; Col-0 in H). Scale bar indicates 50µm.

J: Measuring the minimal and maximal chlorophyll-fluorescence in 3 and 7d old seedlings revealed a decrease in photosynthetic capacity in *RBRcs* seedlings. Asteriks mark significant differences between sugar-treatment in each genotype (*, $p<0.05$; ** $p<0.01$)

Growth arrest of RBRcs seedlings can be partially rescued by sucrose

On medium containing 1% (28mM) sucrose *RBRcs* seedlings displayed a dramatic change in morphology; after 7d most *RBRcs* mutants developed short roots and their cotyledons opened (Figure 4). 14d after germination *RBRcs* seedlings developed a brownish, rugged surface on the hypocotyls and cotyledons, which sometimes turned dark brown (Figure 4). In some cases cells detached from each other producing holes in cotyledons potentially indicating a disturbed cell-to-cell contact (Figure 4 arrowheads). Around 30% of *RBRcs* seedlings displayed outgrowth of undifferentiated structures at the site of the shoot apical meristem (Figure 4, top row, asteriks). After about 5 weeks on culture-medium with sucrose, *RBRcs* seedlings did not develop further. SEM pictures of the surface of cotyledons of 14d old *RBRcs* seedlings demonstrated that the rugged brown surface was the result of massive ectopic cell division activity (Figure 3G and 3H), which likely resumed shortly after germination (Figure 3E and 3F). *RBRcs* seedlings grown on corresponding amounts of glucose showed the same phenotypic abnormalities.

Next we wandered whether this rescue of cell division activity in *RBRcs* mutants on sucrose could be related to sugar signaling or whether it was due to metabolic defects. If it were only signaling effects we would find sugar responses already on low concentrations of sucrose. *RBRcs* seedlings grown on medium containing 0.01% (280nM) sucrose were morphological similar to seedlings grown without sucrose. *RBRcs* mutants grown on medium containing 0.1% sucrose showed first characteristics of sucrose-response, such as opening of cotyledons and changing towards a brownish color (Supplementary figure 2).

Hexokinase1 is a central glucose sensor, which is involved in glucose mediated repression of photosynthetic genes (Rolland et al. 2006). Photosynthesis related gene expression was reduced by low amounts of glucose, but not glucose-6-P in maize protoplasts (Jang and Sheen 1994). *RBRcs* mutants showed a very similar growth on glucose-6-P and glucose (Figure 6D) which could suggest that glucose stimulation of

hexokinase1 was not required for the sugar response that lead to stimulation of cell division.

A low photosynthetic capacity of *RBRcs* seedlings could explain their requirement for external carbohydrates as energy source. Therefore we measured fluorescence of chlorophyll and calculated the ratio of maximal (Fm) and minimal (Fo) fluorescence of individual 3d and 7d old seedlings grown with and without sucrose. *RBRcs* mutants grown with or without sucrose always showed a highly significant reduction of Fm/Fo (Figure 3J) compared to wild type, *RBR-OE* or the hexokinase *gin2/-* seedlings.

In according with a negative effect of sucrose on photosynthesis related gene expression we found a significant reduction of the Fm/Fo ratio in Col-0 seedlings when grown on medium containing 1% sucrose (Figure 3J). Interestingly the Fm/Fo ratio was not reduced in 3d old *RBR-OE* seedlings when grown on sucrose and was even enhanced in 7d old seedlings grown on sucrose similar to the *gin2/-* hexokinase mutant (Figure 3J). These results suggest that *RBRcs* seedlings require external sugars as energy source and indicate a role of RBR in sugar signaling.

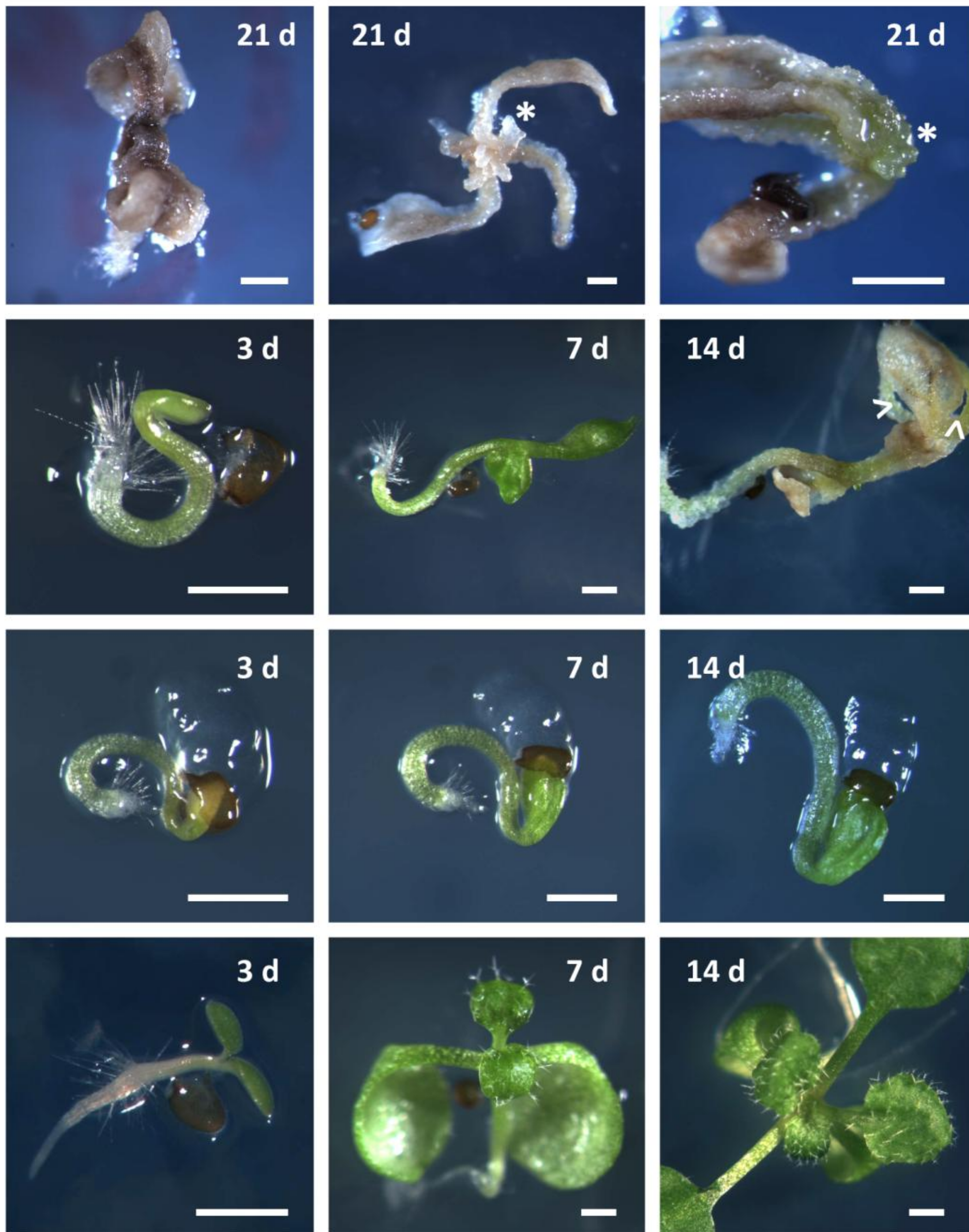


Figure 4.*RBRcs* mutants are developmentally arrested.

Growth comparison of *RBRcs* seedlings grown on medium without or with 1% sucrose. First two rows: *RBRcs* seedlings grown in the presence of 1% sucrose. Third row: *RBRcs* grown without sucrose. After two weeks grown on medium with sucrose, *RBRcs* seedlings develop a brownish rugged surface as a result of massive ectopic cell proliferation. Sometimes cells detach producing wholes in cotyledons (arrowheads, second row) and undifferentiated structures grow out of the original site of the shoot apical meristem (asterisk, first row). When grown without sucrose *RBRcs* seedlings are arrested after an initial stage of organ expansion (third row). At the bottom panel wild type seedlings grown on medium with sucrose are displayed. The scale bare indicates 1mm.

Cell cycle marker genes were highly upregulated in RBRcs seedlings but endoreduplication was decreased

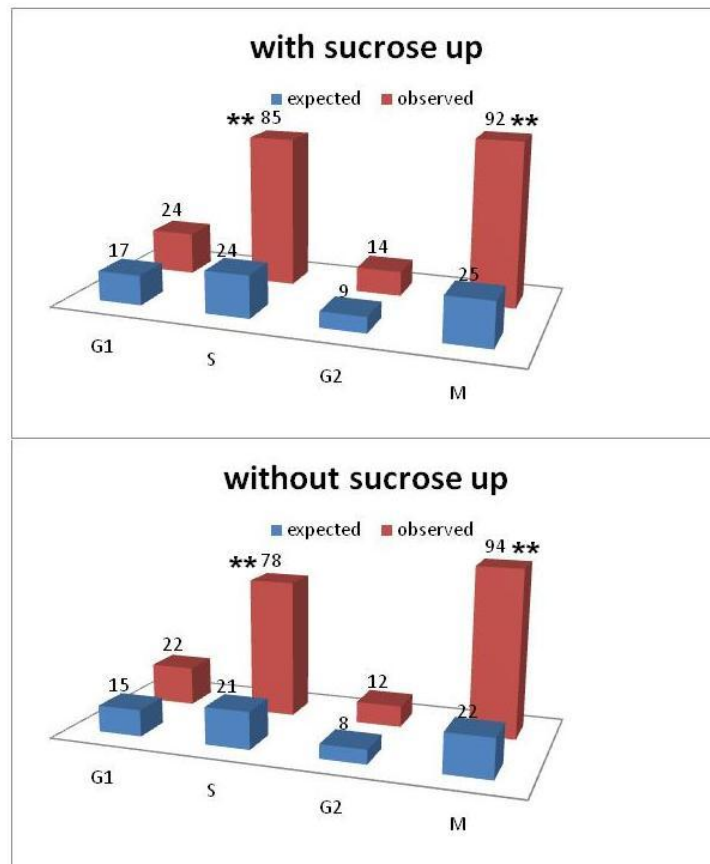
To determine which genes were affected by reduction of RBR and which expression changes might cause the sucrose response of *RBRcs* seedlings, we analyzed the transcriptome of 3d old *RBRcs* and Col-0 seedlings grown with and without 1% sucrose using affymetrix ATH1 microarrays. This early time point after germination was chosen because visible developmental differences between Col-0 and *RBRcs* seedlings were still minimal and *RBRcs* and Col-0 seedlings did not show phenotypic differences when grown with or without 1% sucrose. Statistical analysis of the microarray data identified 2450 and 1939 robustly down and 2266 and 1872 robustly up regulated genes with at least twofold change in *RBRcs* seedlings when compared to Col-0 with and without sucrose respectively. The high number of strongly deregulated genes demonstrated the importance of *RBR* for gene expression homeostasis.

One of the functions of RBR is regulating the G1-S-phase transition by repressing E2F transcription factors, which are necessary for induction of genes important for S-phase progression. Therefore we compared our microarray data with a set of 493 cell cycle specific genes (Menges et al. 2002) and found not only S-phase specific genes, but surprisingly also M-phase specific marker genes significantly enriched in the fraction of upregulated genes in *RBRcs* grown with or without sucrose (Figure 5A). To assess the relative number of cells in different cell cycle phases, we analyzed the DNA content of nuclei from 7 days old etiolated Col-0 and *RBRcs* mutant seedlings via flow cytometric analysis (Figure 5B). In *RBRcs* seedlings grown without sucrose a strong reduction of the number of G2 phase nuclei with 4C DNA content and an accumulation of G1 phase cells with 2C DNA content was found. Together with the previous finding that *RBRcs* seedlings exhibit no post-germinative cell multiplication during hypocotyl expansion, this suggests the absence of DNA replication in this tissue. In *RBRcs* seedlings grown with sucrose the proportion of cells in G2 was rescued but cells in G1 with a 2C DNA content still accumulated (Figure 5B). In both cases endoreduplication was strongly reduced. RBR overexpression had no detectable effect on nuclear DNA content.

When we inspected the nuclear morphology of *RBRcs* in propidium-iodine stained nuclear spreads we found a strong reduction in nuclear size in *RBRcs* mutants (Figure 5C+D, mean: 30µm2+/-14µm2, N=29 vs 78µm2+/-30 µm2, N=20, p<1E-7 for nuclei in the hypocotyl). Consistent with a reduced DNA content, nuclei in hypocotyls cells of *RBRcs* seedlings contained fewer chromocenters (Figure 5D, mean: 6,5+/-1,6 vs. 8,5+/-1,7 for Col-0, N=72, p<1E-10), densely staining heterochromatic regions that correspond to the centromeres and the nuclear organizing regions (Fransz et al. 2002).

From this we conclude that reducing RBR leads to a strong activation of S-and M-phase specific gene expression but to a reduction of nuclear size and endoreduplication.

A



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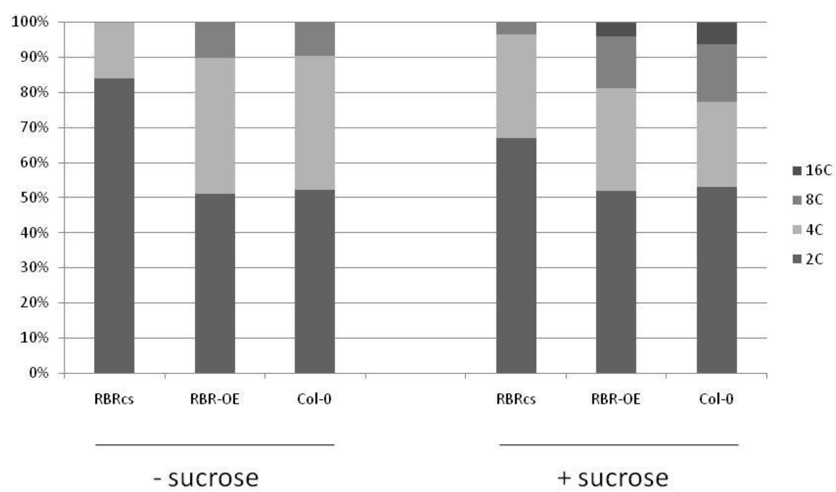


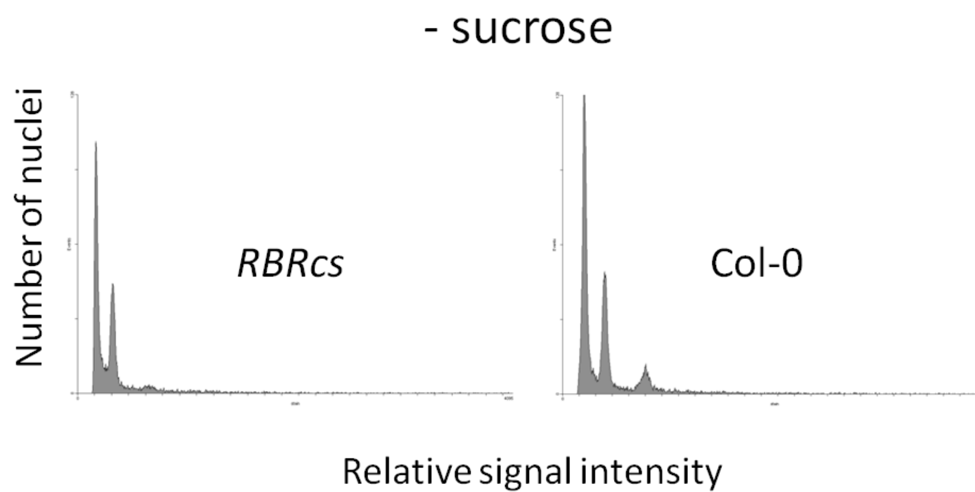
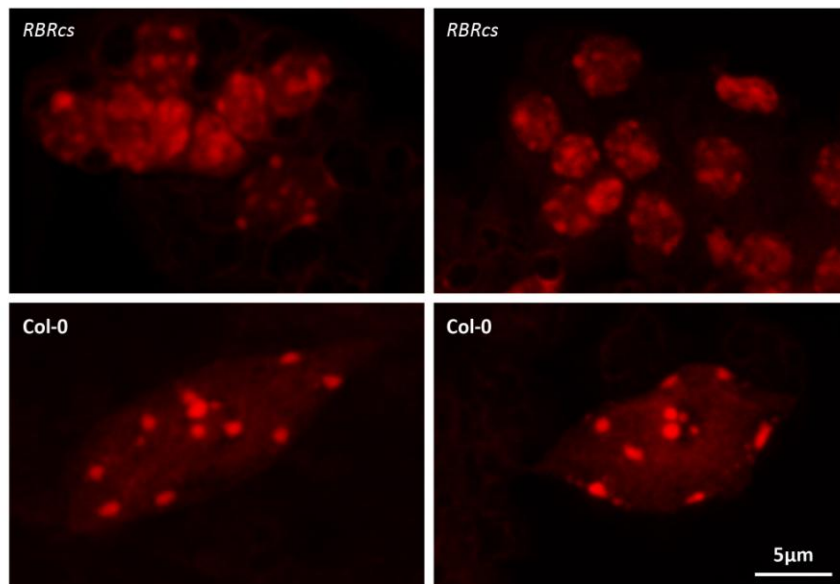


Figure 5.

RBRcs seedlings display enhanced cell cycle activity.

A: In microarray experiments cell cycle marker genes for S and M phase were highly significant enriched in *RBRcs* seedlings (**, p<1E-24). In blue is the theoretically expected number of genes in red the number of genes present in *RBRcs* mutants with increased expression. B: flow-cytometric analysis measures the DNA content of individual nuclei. Displayed is the relative contribution of nuclei with different DNA content. In *RBRcs* seedlings cells with 2C DNA content accumulated and showed less endoreduplication (DNA content >4C) when grown with or without sucrose. Shown is also an example of two flow cytometry histogram plots from Col-0 and *RBRcs* grown without sucrose.

C



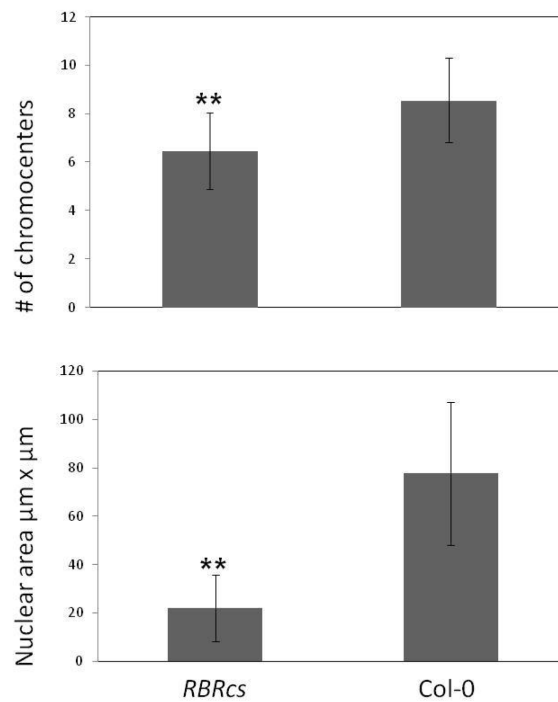


Figure 5.

C: nuclear spreads of 5d old *RBRcs* mutants grown on sucrose show a change in nuclear morphology. On the left are nuclei from the cotyledons, on the right nuclei from the hypocotyl.
D: quantification of nuclear area and of the number of chromocenters showed a highly significant difference between *RBRcs* and Col-0 (**,p<0.01).

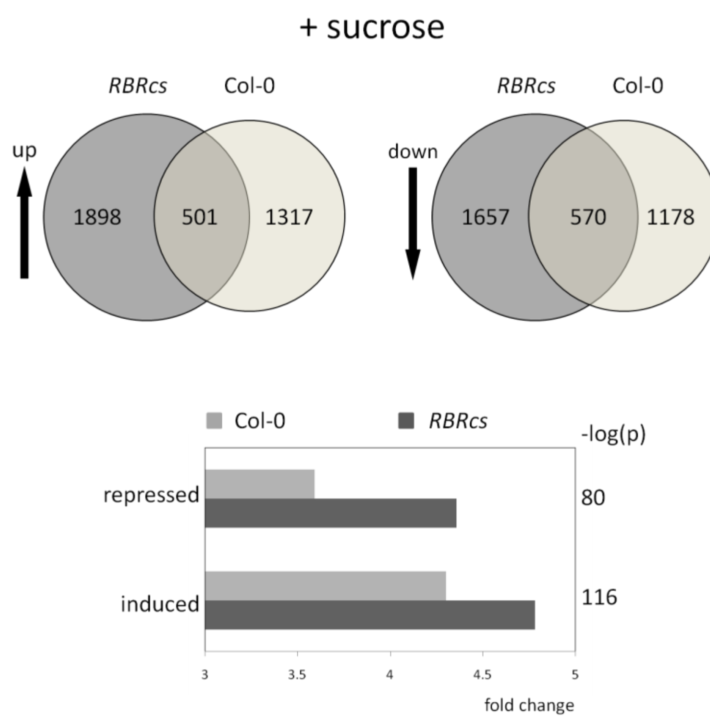
RBRcs seedlings respond strongly to sucrose stimulation

Next we investigated gene expression changes caused by sucrose in the growth medium in 3d old *RBRcs* and wild type seedlings. We found 1317 genes robustly up and 1178 genes robustly down regulated at least 2-fold in response to sucrose in wild type seedlings. In *RBRcs* mutants 1898 genes were robustly induced by sucrose and 1657 genes robustly repressed at least 2-fold. A comparison of *RBRcs* and wild type seedlings grown with and without sucrose revealed that 501 genes were induced and 571 genes repressed significantly at least 2 fold by sucrose in both *RBRcs* mutants and wild type (Figure 6A). The average logarithmic level of induction of these 571 genes was 1.84 in Col-0 and 2.12 in *RBRcs* (p<1E-80) and the logarithmic level of repression of the 501 genes -2.1 in Col-0 and -2.3 in *RBRcs* (p<1E-116) (Figure 6A). This demonstrated that genes, which responded to sucrose in both, *RBRcs* mutants and wild type, changed stronger in the *RBRcs* background suggesting a hypersensitive sucrose-response in *RBRcs* mutants.

Many sugar-hypersensitive mutants, for example the glucose oversensitive (*glo)* (Rolland et al. 2002) or sucrose super sensitive (*sss*) (Pego et al. 2000), are strongly germination-inhibited in the presence of low concentrations of sucrose. We did not find this effect in *RBRcs* mutants and germination kinetics was comparable to wild type up to 3% sucrose in the growth medium (data not shown). Interestingly many sugar transporter displayed elevated expression in *RBRcs* seedlings grown with but also without sucrose, suggesting that *RBRcs* seedlings were primed for the take up of sugars for heterotrophic growth (Figure 6C).

Next we measured internal sugar and starch concentrations of *RBRcs* and Col-0 seedlings. We found significant less starch in 7d old *RBRcs* mutant seedlings grown without sucrose and significantly more sucrose and starch in *RBRcs* mutants grown with 1% sucrose compared to 7d and 3d old control seedlings (Figure 6B). The content of fructose and glucose was not significantly changed.

In order to reveal potential cell-identity changes towards heterotrophy in *RBRcs* seedlings grown with or without sucrose we performed a principal component analysis of 5% genes (1160 genes) with highest expression-variation in our microarray data set plus data from 27 different tissues of *Arabidopsis* (Schmid et al. 2005). Figure 7A shows a two-dimensional projection of the first 3 principal components. 3d and 8d old wild type seedlings showed only a very small shift in the first three components when grown on medium with 1% sucrose (arrowheads). *RBRcs* seedlings showed a dramatic shift in gene expression towards heterotrophic tissues like root and surprisingly, maturing seeds (arrow).


A
+ sucrose
RBRcs
Col-0
up
1898
501
1317
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down
1657
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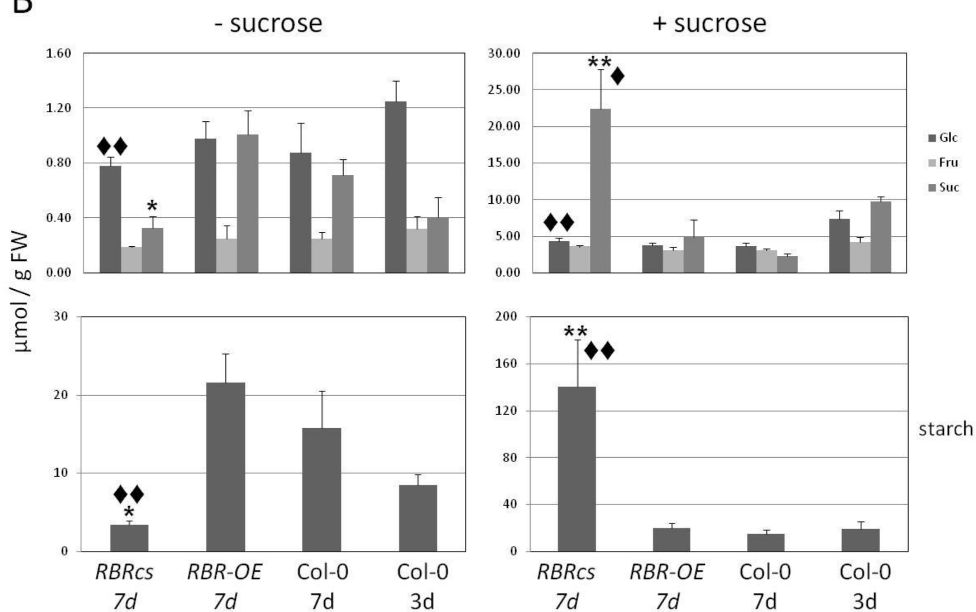
B
- sucrose
+ sucrose
µmol / g FW
Glc
Fru
Suc
starch
RBRcs 7d
RBR-OE 7d
Col-0 7d
Col-0 3d

Figure 6. *RBRcs* seedlings responded strongly to sucrose stimulation.

A: Shown is the overlap of genes that were either induced or repressed by sucrose in *RBRcs* and Col-0. On average sucrose inducible or repressible genes responded stronger in the *RBRcs* background (bottom).

B: *RBRcs* seedlings can readily take up sucrose. 7d old *RBRcs* seedlings consist mainly of cotyledons and the hypocotyls, therefore we used 3d and 7d old Col-0 seedlings as controls. When grown on medium without sucrose, *RBRcs* seedlings contained significant less starch than Col-0 seedlings. When grown on medium containing 1% sucrose *RBRcs* seedlings accumulated significant more sucrose and starch than all controls (♦ p<0.05, ♦♦ p<0.01 compared with 3d old Col-0 seedlings; * p<0.05, ** p<0.01 compared with 7d old Col-0 seedlings).

C

locus	annotation	change - sucrose	p-value - sucrose	change + sucrose	p-value + sucrose
AT5G57100	glucose-6-phosphate translocator-like	2.6	5.9E-13	2.0	3.5E-06
AT5G10190	carbohydrate transmembrane transporter activity	2.8	2.5E-07	2.4	5.3E-03
AT1G09960	SUCROSE TRANSPORTER 4	2.0	3.3E-03	3.9	6.1E-18
AT3G05400	sugar transporter	4.0	1.2E-06	2.0	8.1E-03
AT4G17550	similar to glycerol-3-phosphate translocator	2.2	3.2E-04	4.3	8.5E-07
AT5G59740	UDP-galactose/UDP-glucose transporter-related	3.3	1.9E-11	3.6	4.9E-09
AT1G79820	SGB1; carbohydrate transporter	3.1	1.1E-06	4.1	5.0E-09
AT3G20660	carbohydrate transporter	2.5	1.6E-03	8.1	6.5E-07
AT1G71890	SUCROSE-PROTON SYMPORTER 5	11.0	9.8E-13	3.2	2.0E-03

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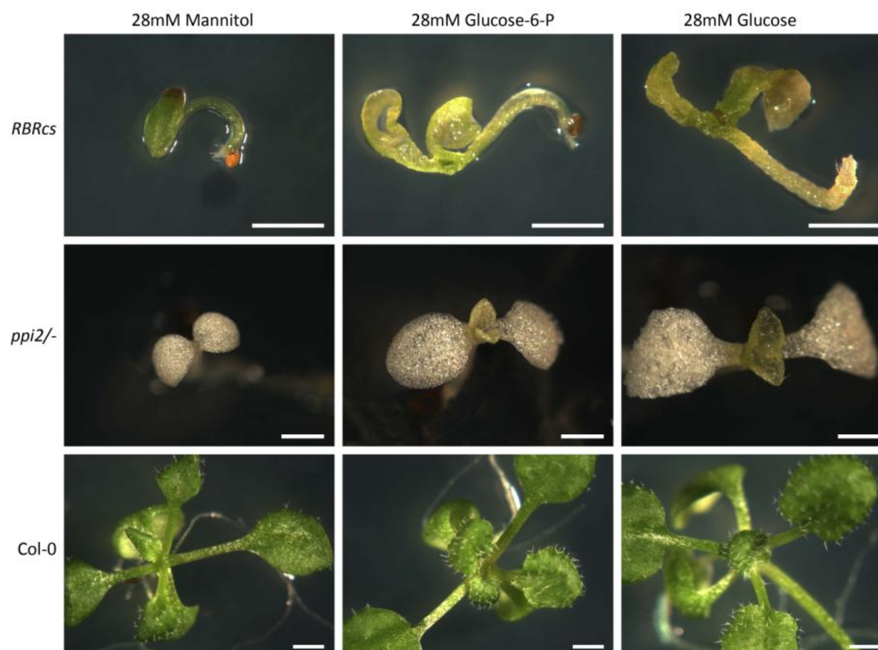


Figure 6. *RBRcs* seedlings responded strongly to sucrose stimulation.

C: sugar transporters with increased expression in *RBRcs* grown with or without sucrose

D: phenotypic abnormalities of 14d old *RBRcs* seedlings grown on medium supplemented with 28mM glucose are similar to seedlings grown with 28mM glucose-6-phosphate; the heterotrophic plastid-import-mutant *ppi2*/- demonstrated that glucose-6-phosphate can be taken up efficiently.

RBRcs seedlings display strong sugar dependent derepression of embryo specific genes

In order to determine, which genes may be responsible for the sugar response in *RBRcs* seedlings, a hierarchical clustering of the 100 strongest induced genes in *RBRcs* mutants was performed with the genevestigator (Zimmermann et al. 2004) anatomy tool. Figure 7B shows that from these 100 genes, a cluster of 67 genes is specifically expressed in seed, imbibed seed, endosperm and embryo. None of these genes changed expression in response to sucrose in Col-0 seedlings. Furthermore from this cluster of 67 genes, 40 genes were not deregulated in *RBRcs* seedlings grown without sucrose when compared to Col-0 seedlings (supplementary table 2). All 40 can be classified as embryo-specific genes and the group was strongly enriched in genes involved in seed maturation, including seed storage proteins like oleosin2, two cruciferins, a cupin family protein and 7 late embryogenesis abundant proteins. All these genes changed expression dramatically from 30 up to more than 400 times. To confirm the accumulation of the respective gene products, proteins from 3d old *RBRcs* and Col-0 seedlings grown on medium with 1% sucrose were extracted and subjected to SDS page (Figure 7C). After tryptic digest of gel slices of the respective size and subsequent tandem mass spectrometry we were able to identify 33 proteins that were encoded by the 40 previously identified embryonic specific expressed genes. All showed a strong accumulation with respect to total number of tryptic peptides and APEX factor (Lu et al. 2007) (Figure 7C).

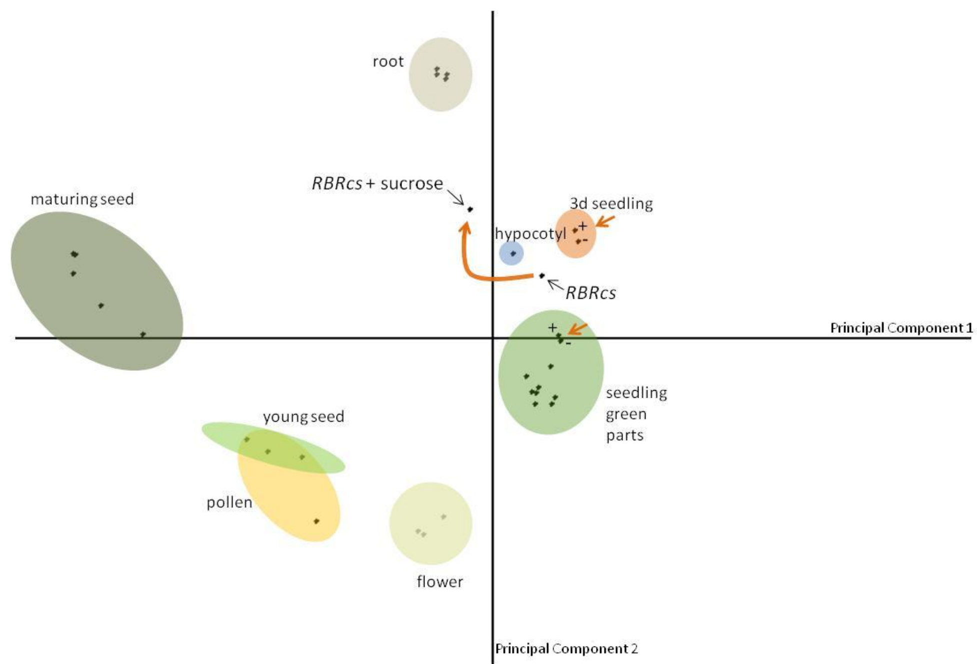
Among the sugar-induced embryonic and seed maturation genes in *RBRcs* we also found the transcriptional regulators abscisic acid insensitive 3 (*ABI3*) and 5 (*ABI5*). *ABI3* is known to induce seed maturation genes upstream of *ABI5* and is likely repressed later in sporophyte development by polycomb-group complexes via methylation of histone H3K27 in its promoter region (Lopez-Molina et al. 2002; Nakashima et al. 2006; Zhang et al. 2007). From 14 published genes that were induced in an *ABI3* overexpressing line (Nakashima et al. 2006) 10 were upregulaten in *RBRcs* plus sucrose but none in *RBRcs* without sucrose. Of these, 7 had transcript levels more than 30 times higher than wild type (supplementary table 3).

ABI3 belongs to plant specific transcription factors with a B3 DNA-binding domain. B3 domain transcription factors are involved in regulating sugar response, embryo development, seed maturation and germination. The B3 domain binds to a specific DNA sequence, the RY motiv. Promoter analysis of the strongest induced genes in *RBRcs* mutants with or without sucrose revealed a highly significant enrichment of the RY motive, suggesting a regulation of these genes via B3-domain transcription factors (Figure 7D). The genome of *Arabidopsis* contains 6 B3 transcription factors, *ABI3, LEC2, FUS3, HSI2, HSL1* and *HSL2.* To confirm the potential sucrose inducibility of *ABI3, ABI5* other B3 transcription factors and two potential targets genes of ABI3 in the *RBRcs* background we measured transcript levels during a time-course in *RBRcs* and Col-0 seedlings; *RBRcs* and Col-0 seedlings were germinated on normal MS medium and transferred 3 days after germination to medium with or without 1% sucrose. Quantitative PCR showed that the levels of *HSI2, HSL1* and *FUS3* were not significantly different in *RBRcs* seedlings compared to Col-0 seedlings independent of sucrose treatment (Figure 8). *HSL2* was slightly reduced in *RBRcs* seedlings, whereas the levels of *ABI3* and *LEC2* expression were strongly increased already at 3d after germination without sucrose. After transfer to medium with sucrose, the levels of *LEC2* and *ABI3* increased even more, demonstrating a strong additional induction by sucrose (Figure 8). Although the levels of *ABI3* expression decreased later again, it was still higher compared to wild type. Very similar kinetics were found also for the expression of *OLES2* and a gene annotated as abscisic acid responsive gene, which contain several RY motives in their promoters and for *ABI5*.

Consistent with derepression of late embryonic specific genes in *RBRcs* mutants grown on sucrose we found an accumulation of neutral lipids in *RBRcs* seedlings, which do usually accumulate only in wild type mature embryos. In *RBRcs* seedlings grown on medium with sucrose we found already accumulation of neutral lipids in cotyledons (Figure 9A,A) 7d after germination and a conspicuous staining in 14d old seedlings (Figure 9 A,B).

Taken together, these results suggest that RBR is important for proper cell specification in *Arabidopsis* after germination by repressing a late embryonic, sugar inducible transcriptional programs.

A



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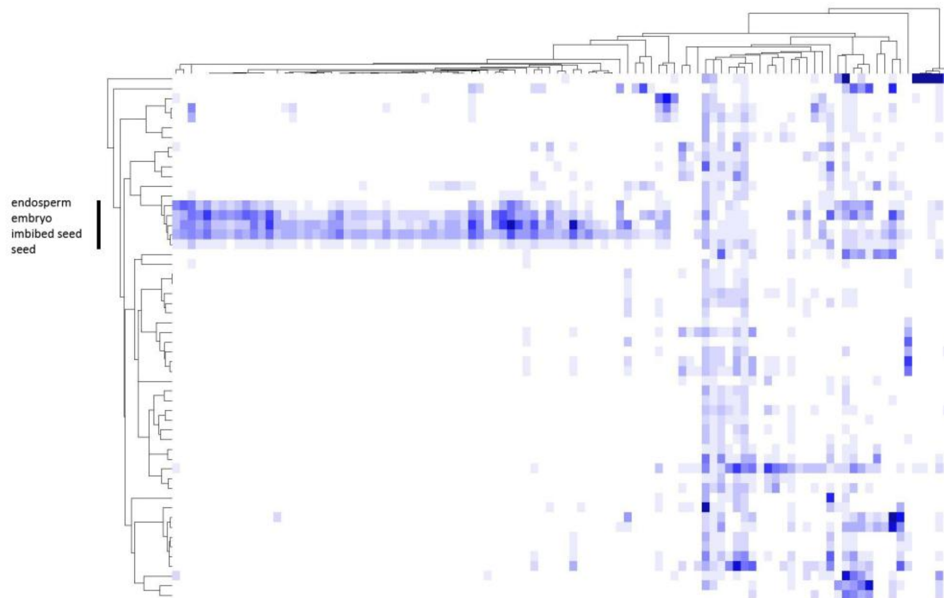
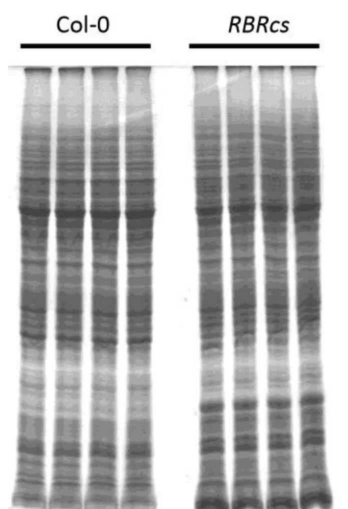


Figure 7.

A: Principal component analysis of 5% genes with the highest variation amongst different *Arabidopsis* tissue and *RBRcs* plus control seedlings. Addition of sucrose caused only a minor shift in gene expression in 3d and 8d old Col-0 seedlings (arrowheads) but a major shift towards maturing seedlings and roots in *RBRcs* seedlings.

B: hierarchical clustering of the 100 genes with strongest differential expression in *RBRcs* reveals a conspicuous cluster specific for embryo, endosperm, imbibed seed and seeds.

C



atg-number	Annotation	Col-0 tot num	RBRcs tot num	Col-0 apex	RBRcs apex	ATH1 change (log)
AT3G01570	glycine-rich protein / oleosin	19	38	11.3	25.8	7.7
AT5G03860	MLS (MALATE SYNTHASE); malate synthase	84	97	8.4	11.0	7.4
AT5G40420	OLEO2 (OLEOSIN 2)	16	33	5.2	12.2	8.7
AT4G28520	CRU3 (CRUCIFERIN 3); nutrient reservoir	28	331	3.0	40.9	5.0
AT1G33790	jacalin lectin family protein	15	40	2.1	6.3	5.6
AT4G16160	ATOEP16-2 protein transmembrane transporter	5	15	1.6	5.6	7.9
AT5G44120	CRA1 (CRUCIFERINA); nutrient reservoir	12	212	1.4	28.8	7.2
AT1G17810	BETA-TIP, water channel	4	8	1.2	2.7	5.9
AT1G54870	oxidoreductase	6	43	1.0	8.4	5.9
AT1G48130	ATPER1, antioxidant	4	17	0.9	4.3	8.9
AT2G34700	pollen Ole e 1 allergen and extensin family protein	3	7	0.9	2.4	4.9
AT1G65090	similar to unknown protein	3	7	0.7	1.8	7.5
AT2G47780	rubber elongation factor (REF) protein-related	2	5	0.5	1.4	9.3
AT5G22470	NAD+ ADP-ribosyltransferase	3	18	0.2	1.4	4.8
AT1G52690	late embryogenesis abundant protein	0	3	0	0.9	8.3
AT3G15670	late embryogenesis abundant protein	0	14	0	3.6	7.9
AT1G48660	auxin-responsive GH3 family protein	0	5	0	0.6	7.4
AT3G17520	late embryogenesis abundant	0	11	0	2.6	7.3
AT5G06760	late embryogenesis abundant	0	2	0	0.9	7.3
AT3G22640	cupin family protein	0	137	0	18.6	7.0
AT3G53040	late embryogenesis abundant protein, putative	0	4	0	0.5	6.8
AT5G07330	similar to unknown protein	0	3	0	0.9	6.8
AT1G15330	CBS domain-containing protein	0	4	0	1.1	6.7
AT2G28490	cupin family protein	0	26	0	3.8	6.3
AT5G24130	similar to unnamed protein product	0	2	0	0.6	6.2
AT5G01870	lipid transfer protein, putative	0	4	0	3.3	6.2
AT1G64110	AAA-type ATPase family protein	0	5	0	0.3	6.2
AT1G05490	CHR31 (chromatin remodeling 31)	0	4	0	0.1	5.8
AT2G36640	ATECP63 (EMBRYONIC CELL PROTEIN 63)	0	9	0	1.1	5.7
AT2G31980	cysteine proteinase inhibitor-related	0	2	0	0.7	5.6
AT5G24280	ATP binding	0	3	0	0.1	5.4
AT3G11050	ATFER2 (FERRITIN 2); ferric iron binding	0	7	0	1.9	5.1
AT1G13330	similar to unnamed protein product	0	2	0	0.6	4.8

D

	motif	number of motifs	number of sequences	p-value
100 strongest up + sucrose	ABRE (ACGTGG)	63	38/100	2.70E-10
	RY (CATGCA)	57	41/100	5.26E-09
100 strongest up	ABRE (ACGTGG)	19	14/100	1.10E-01
	RY (CATGCA)	22	20/100	6.77E-02

Figure 7.

C: SDS-page of protein extract prepared from 3d old *RBRcs* and Col-0 seedlings grown on sucrose. The bar indicates the region of the gel we found late embryonic proteins (table).

D: The RY motiv is highly significantly enriched in promoter regions of the strongest upregulated genes in *RBRcs* mutants grown with sucrose.

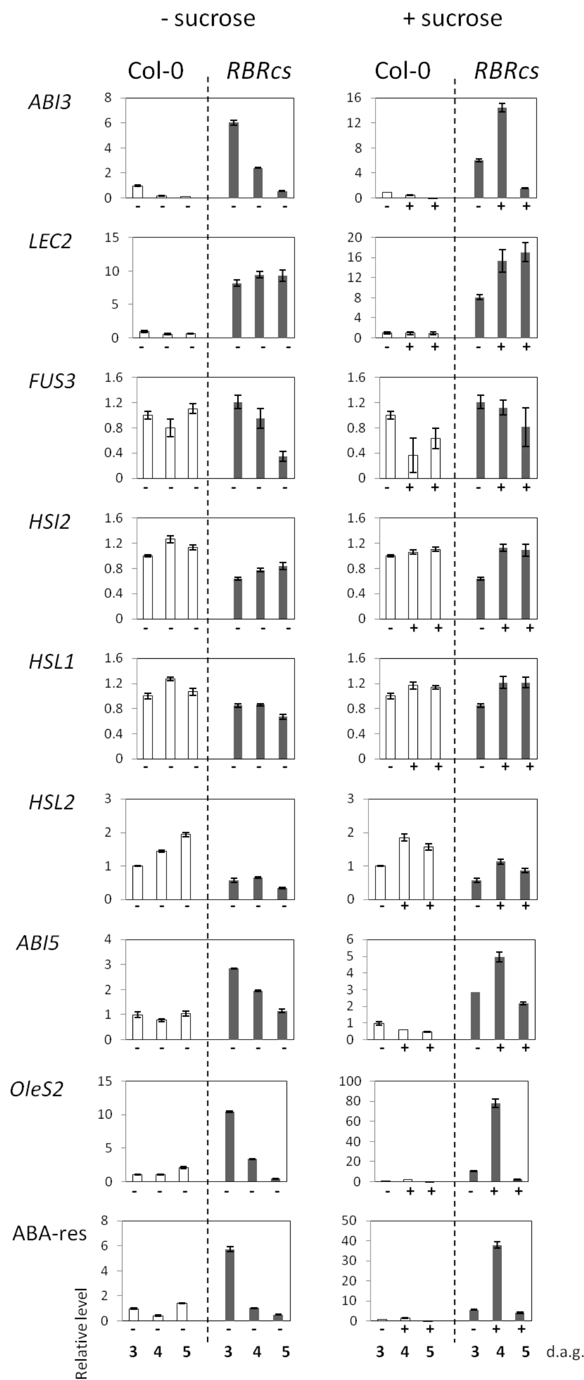


Figure 8.

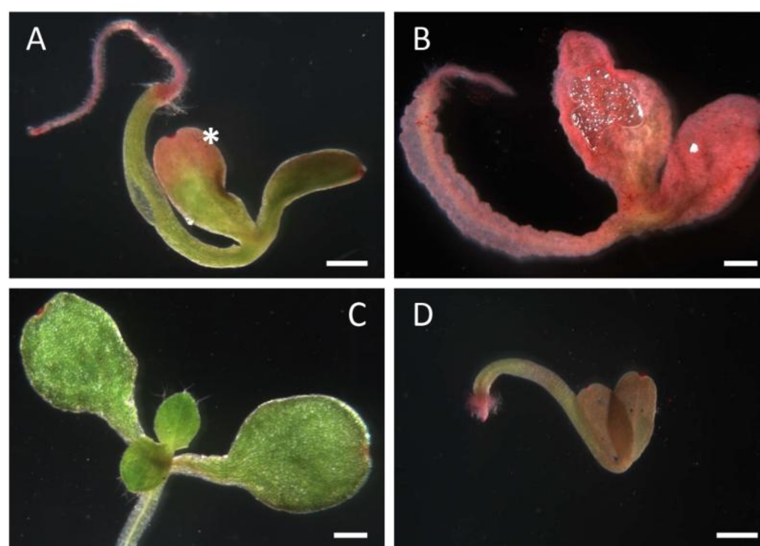
To test sucrose inducibilty of selected genes, *RBRcs* and Col-0 seedlings were germinated on medium without sucrose and transferred on the third day to medium either with 1% (+) or without (-) sucrose. From the B3 domain transcription factors, *LEC2* and *ABI3* responded strongly to the sucrose stimulation in *RBRcs* mutant background but not in Col-0 seedlings. Also *ABI5*, *OLES2* and a gene annotated as abscisic acid responsive gene showed a strong induction upon sucrose stimulation.

RBR expression can be induced by sucrose in early germinating seedlings

Although we were not able to detect sucrose dependent induction of embryonic genes in 3d old Col-0 seedlings, the presence of 1% sucrose in the growth medium caused a slight delay in degradation of embryonic oil-bodies from the second to the third day after germination (Figure 9B and supplementary figure 3). This could indicate that sucrose inhibits the repression of the embryonic program also in Col-0 seedlings early after germination, which could explain the inhibitory effects of sucrose on seed germination. If RBR was important to repress late embryonic gene expression, *RBR* subsequently could be induced by sucrose in wild type seedlings early after germination to allow cells to progress from a heterotrophic to an autotrophic identity when they start photosynthesis. Therefore we performed a time-course analysis of *RBR* promoter activity with a *GUS*-reporter in seedlings grown either without or with 1% or 3% of sucrose. Figure 9C shows that there was no difference in *GUS* expression one day after germination on different sucrose concentrations. Two days after germination *GUS* expression was strongest in seedlings grown on 3% sucrose but also seedlings grown with 1% or without sucrose displayed strong GUS-staining in the cotyledons. 3 days after germination GUS staining of cotyledons in seedlings grown without sucrose was confined to the vasculature. In seedlings grown on 1% or 3% sucrose GUS staining was still strong in cotyledons. Four days after germination only seedlings grown on 3% sucrose displayed strong *GUS* activity and seven days after germination there was no difference in GUS staining of seedlings grown on different sucrose concentrations. Western-blot analysis and quantitative pcr of 3d old seedlings confirmed the GUS-staining pattern (Figure 9D).

These results could indicate that in young seedlings, which germinate under very favorable conditions and hence can start photosynthesis early, *RBR* could be induced by sucrose to prevent reactivation of embryonic gene expression.

A



B

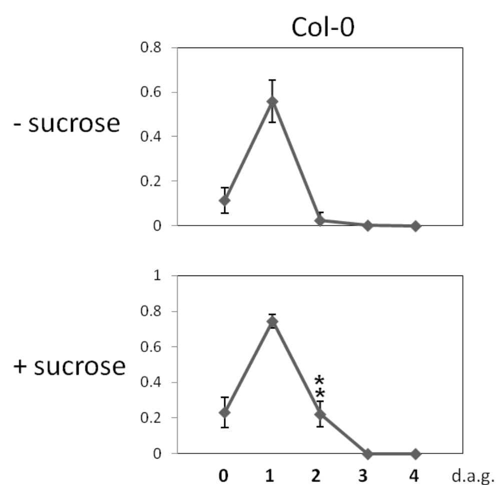
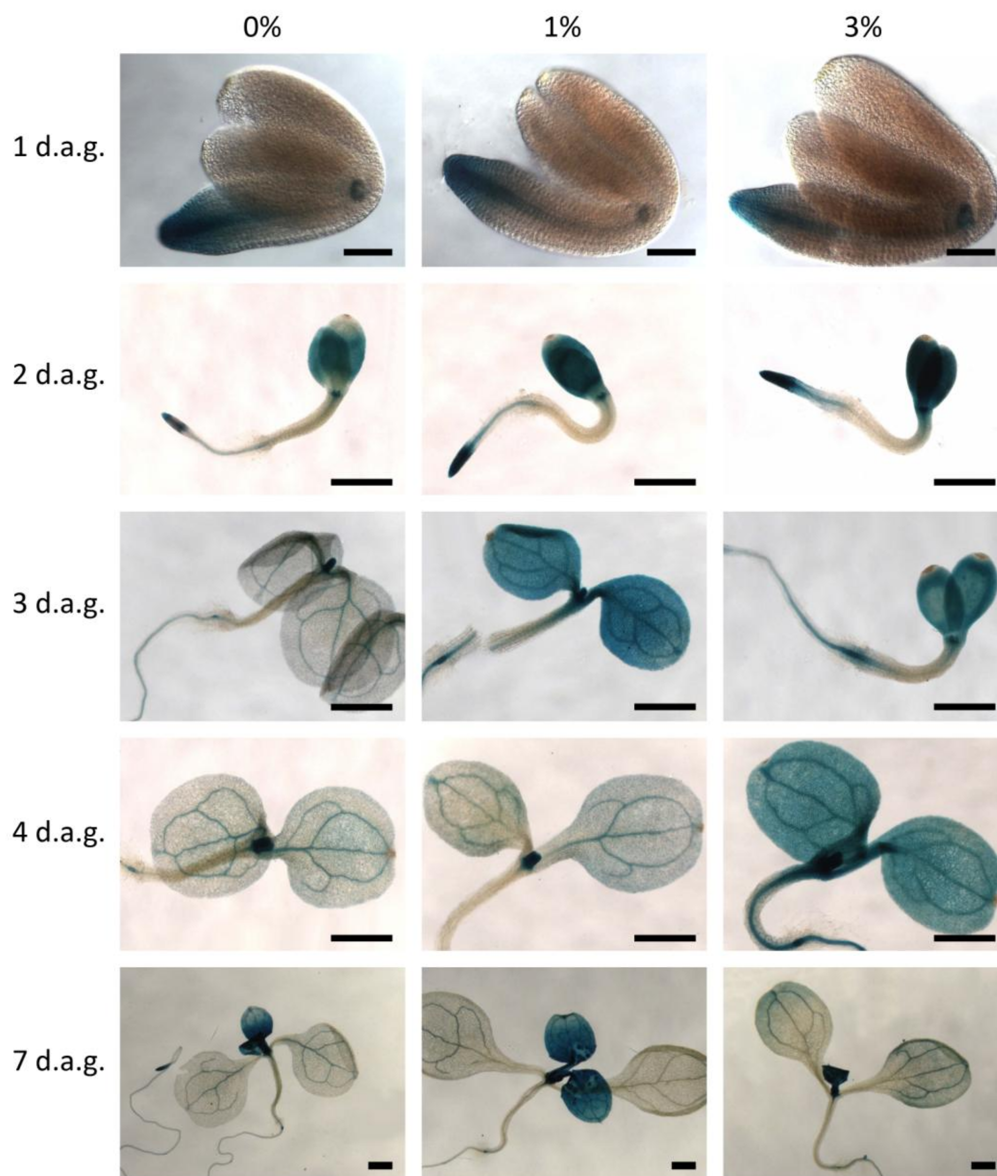


Figure 9.

A: Sudan red stains neutral lipids that usually only accumulate in mature embryos. Seven day old *RBRcs* mutants grown on medium with 1% sucrose start to accumulate lipids at the tip of cotyledons (A asterisk). 14 day old seedlings grown on sucrose accumulated a conspicuous amount of neutral lipids (B) compared to mutant seedlings grown without sucrose (D) or 7d old wild type seedlings on sucrose (B).

B: sucrose causes delay of degradation of embryonic oil-bodies. Shown is the ratio of area occupied by embryonic oil bodies compared to the whole area (see also supp. Figure 4). Seedlings grown on sucrose accumulate more oil-bodies and need longer to metabolize them (**, $p<0.05$).

C



D

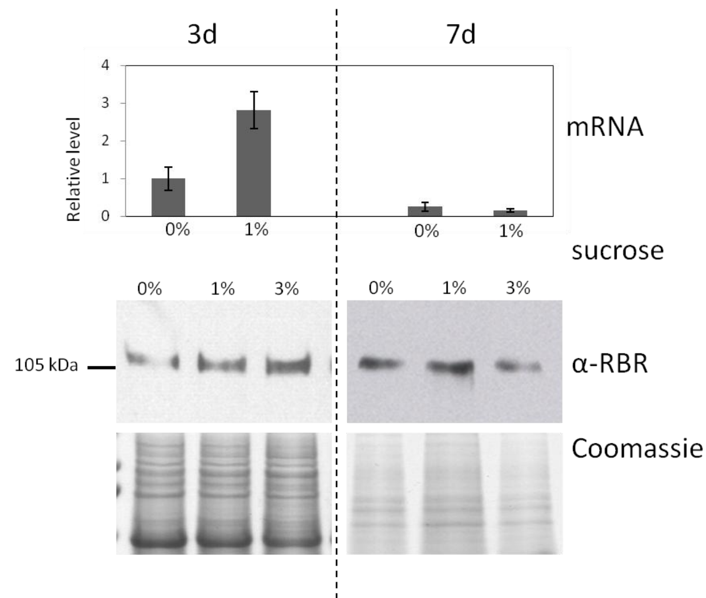


Figure 9.

C+D: sucrose induces *RBR* in early germinating seedlings. *RBR* promoter GUS activity is stronger in 2d, 3d and 4d old seedlings grown on medium supplemented with 1% and 3% sucrose. In 7d old seedlings there is no conspicuous difference. D: This pattern was confirmed by western blot analysis and quantitative PCR of 3d and 7d old seedlings grown on different concentrations of sucrose. 3d old seedlings showed higher expression of *RBR* when grown on 1% sucrose and higher levels of RBR protein on medium with 1% and 3% sucrose. In contrast *RBR* transcript or protein was not inducible by sucrose in 7d old seedlings.

Discussion

During sporophyte development, plants undergo several phase transitions. After fertilization, the zygote develops to produce a mature embryo, which becomes desiccation tolerant end enters a dormant stage. After this stage the embryo can germinate when conditions are favorable. During seedling establishment, all cells of the seedling start with a heterotrophic, embryonic stage at which storage reserves are metabolized. Subsequently most cells start photosynthesis and switch towards an autotrophic lifestyles. These processes are controlled by the integration of external signals with intrinsic genetic programs (Suzuki and McCarty 2008).

Post-embryonic plant development is directed by populations of stem cells that produce defined patterns of cell proliferation in the shoot and root apical meristems. Finally, seedlings change from a juvenile growth to an adult growth in which they become competent to induce flowering.

The mechanisms linking cell proliferation, cell fate switch and hence differentiation are still not well understood and it is not known whether phase transition-events have common molecular characteristics and how this is connected to cell cycle regulation. pRB seems to link cell cycle regulation with developmental aspects in animals, thus being an interesting candidate for a global integrator of cell cycle into plant development. However the study of RBR in plant development has been not trivial because loss of RBR function in Arabidopsis is gametophytically lethal (Ebel et al. 2004) and recent attempts to reduce *RBR* expression with RNA interference technology have failed (Jullien et al. 2008).

We could show that although RBR is expressed in all plant tissues investigated, the levels of RBR protein were strongly increased in proliferating and young tissue compared to fully expanded and differentiated tissue. This suggests that rather than simple switching on a break in the cell cycle, RBR is important to regulate cell division. After cells have entered Go phase and are fully differentiated, low levels of RBR could be sufficient to repress re-entry into cell cycle. Alternatively it could be possible that the activity of RBR is changed via post-transcriptional modifications in different cell types. For example in pea it has been demonstrated that RBR becomes hyperphosphorylated in axillary buds during the transition from dormancy to growth (Shimizu-Sato et al. 2008). Development of tools for the analysis of cell or tissue specific phosphorylation states of RBR will be required to adress this question in *Arabidopsis*.

In order to investigate the role of RBR for plant development we generated *35sRBRi* and *RBRcs* lines that had strongly reduced levels of RBR protein. Interestingly sister plants of

RBRcs seedlings with increased levels of RBR did not show any discernable phenotype. That could mean that either the amount of additionally produced active protein was not sufficient to cause defects in cell cycle regulation or that RBR-levels were not changed in cells potentially susceptible to higher RBR levels. During embryogenesis we were not able to distinguish *RBRcs* from wild type embryos until the bent cotyledon stage. In this stage embryos that would give rise to the strong seedling-lethal phenotpye showed an increased number of cells. Additional cells in *RBRcs* embryos may be the result of a shortening of the cell cycle or a prolonging of the proliferative phase (De Veylder et al. 2002). Component of the silencing machinery are important early in embryo development (Lynn et al. 1999; Kerstetter et al. 2001; Schauer et al. 2002). Therefore transgene silencing and co-suppression could also start early in embryo development. Alternatively, we could probably not distinguish *RBRcs* from wild type embryos earlier, because RBR levels were reduced only late in embryo-development. Due to the inverse correlation of *RBR* message and protein in *RBRcs, 35sRBRcs* and *35sRBRi,* only protein measurement was reliable to quantify RBR levels. Therefore *in situ* hybridization would not have been useful to infer *RBR* activity early in embryo development.

All seedlings with reduced RBR levels germinated but were arrested in development. Interestingly although the seedlings were green and cell cycle genes were strongly upregulated there was no cell division activity in *RBRcs* seedlings without external sugar-supply. In contrast to many reports that demonstrated an increase in endoreduplication when manipulating the activity of RBR (De Veylder et al. 2002; Park et al. 2005; Desvoyes et al. 2006) or overexpressing E2Fa and DPa (De Veylder et al. 2002) we found the opposite, a decrease in endoreduplication in *RBRcs* seedlings grown either with or without sucrose. Interestingly in *RBRcs* seedlings not only S-phase genes, which are enriched in E2F-sites in their promoters but also M-phase genes that do not carry E2F-sites in their promoters showed a highly significant increased expression. That could mean that the differences in the endoreduplication state that we observed was due to an increased mitosis in late embryogenesis, or that the effect of downregulating RBR on endoreduplication is different in embryos and young seedlings than in leaves. Although unlikely in the case of *RBRcs* seedlings grown with sucrose, the lack of endoreduplication in *RBRcs* mutants grown without sucrose might simply be an insufficient energy-supply.

Endoreduplication has also been used as a marker for the differentiation state of cells and in leaves it occurs only after the cessation of normal mitotic cycles (De Veylder et al. 2001). Interestingly, leaf cells of *CYCD3;1* overexpressing seedlings were also deficient in endoreduplication (Dewitte et al. 2003). Since D-type cyclins are potentially phosphorylating RBR, which results in a disruption of RBR/E2F complexes (de Jager and Murray 1999) it could be that lack of endoreduplication in *RBRcs* and *CYCD3;1* overexpressing seedlings is a result of low RBR activity. *CYCD3;1* as well as *E2Fa-DPa*

overexpressing seedlings displayed strong developmental defects and overproliferation in leaf cells (De Veylder et al. 2002; Dewitte et al. 2003). However *RBR* mRNA and protein was increased in *CYCD3;1* overexpressing seedlings (Dewitte et al. 2003). In accordance with reduced endoreduplication in *RBRcs* seedlings this would suggest that RBR was inactive in *CYCD3;1* overexpressing seedlings and that active RBR is promoting cellular differentiation independent of the *E2Fa-DPa* pathway.

Very different to the situation in animals, plants consist of source tissues that produce and export sugars such as rosette leaves, and sink tissues that imports sugars such as embryos, roots and the shoot apical meristem. Sucrose is not only the transportable form of the chemically fixed energy from photosynthetic to sink tissue but probably the most important signaling molecule regulating interactions between these two different types of tissue. Plant hormones play important roles in fine-tuning sink-source interactions. In recent years, the importance of sugars in plant growth and development and key players in the sugar-signaling network have been uncovered (Rolland et al. 2006). Surprisignly it has been found that a fraction of the hexokinase pool resides in the nucleus (Cho et al. 2006) and can directly regulate gene-expression. Glucose-6-phosphate can circumvent hexokinase signaling and in maize protoplasts low amounts of glucose but not glucose-6-phosphate reduced photosynthesis related gene expression, (Jang and Sheen 1994). *RBRcs* seedlings on medium containing glucose-6-phosphate or glucose showed similar phenotypic abnormalities. This could indicate that the strong effects that we observed were independent of hexokinase signaling.

However most of the transcriptional modifiers that have been associated with sucrose signaling do not have homologs in animals and are often involved in seed and seedling development.

Here we give evidence that RBR is an important regulator of the sugar response in germinating *Arabidopsis* seedlings. Seedlings with reduced RBR levels had a hypersensitive expression response to sucrose and behaved in many respects like sink tissue: sugar-transporters were upregulated in combination with a highly increased tendency to take up sucrose, photosynthetic capacity was reduced and cell division was strongly induced by sugars.

These findings raise the questions whether similar regulations exist in other than embryonic sink tissue such as the shoot apical meristem. An attractive hypothesis would be that a reduced activity of RBR would be necessary to maintain cell division in response to sugar signals and once cells start to be committed to a particular determination, RBR would be necessary to repress activity of genes that maintain an undifferentiated state.

Our results further show that *RBRcs* seedlings are defective in going through the phase transition from heterotrophic embryonic-, to autotrophic seedling-growth. Differential gene expression analysis demonstrated that only in the presence of sucrose embryonic marker genes and especially seed maturation genes were strongly induced in *RBRcs* seedlings, but not in wild type seedlings. Interestingly a double mutant of two B3-domain transcription factors showed a very similar phenotype to *RBRcs* mutants (Suzuki et al. 2007; Tsukagoshi et al. 2007). Seedlings of this *kk* mutant were arrested after germination but upon addition of 1% sucrose hypocotyls developed into yellow callus-like structures. *kk* mutants also showed a derepression of seed maturation genes. However the levels neither of hsi2 nor of hsl1 were significantly reduced in *RBRcs* mutants. Instead, the expression of *ABI3, ABI5* and *LEC2* transcription factors, which are involved in embryonic growth, seed maturation and embryo to seedling transition, changed significantly. Since *ABI3* is upstream of *ABI5* (Lopez-Molina et al. 2002) it is tempting to speculate that RBR is modifying the promoter activity of *ABI3* and *LEC2* to prevent its sugar inducibility. Interestingly the promoter histones of *ABI3* are strongly methylated by the repressive H3K27me3-mark (Zhang et al. 2007) and the association of RBR with polycombgroup-proteins, which form this histone modification has been demonstrated (Ach et al. 1997; Mosquna et al. 2004; Guitton and Berger 2005; Jullien et al. 2008). Consistently, mutants for the polycomb-group gene *EMF2* show a derepression of seed maturation genes when grown on medium containing sucrose (Moon et al. 2003). The plant homolog of the retinoblastoma-binding protein 48 – MSI1- is part of the medea/fertilization independent endosperm polycomb group complex and is required for seed development (Kohler et al. 2003). Recently the interaction of MSI1 with RBR has also been demonstrated in Arabidopsis (Jullien et al. 2008).

Another potential chromatin modifying factor that is important to repress embryonic traits after germination is *PICKLE* (Ogas et al. 1997; Li et al. 2005). *PICKLE* belongs to the CHD3 group of SWI/SNF class chromatin remodeling factor (Eshed et al. 1999; Ogas et al. 1999), proteins that have been described as repressors of developmentally regulated genes by recruiting histone-deacetylases (Kehle et al. 1998). The primary roots of *pickle* seedlings accumulated seed storage lipids and inhibition of GA biosynthesis resulted in increased penetrance of the phenotype (Ogas et al. 1997). Although we did not detect expression changes of *PICKLE* in *RBRcs* seedlings (data not shown), this does not exclude the possibility that RBR and PICKLE act together to repress embryonic traits after germination and thus allow cell fate switches towards an autotrophic lifestyle.

Deciphering patterns of changes in chromatin and identifying the action of the most important proteins that are involved might lead to a general understanding of how cells are able to change their identities.

Material and methods

Plant material and growth conditions

Construction of lines with reduced levels of RBR: to produce *35S::RBRcs* seedlings the whole genomic sequence of RBR was fused to the cauliflower mosaic virus (CaMV) 35S promoter into the binary vector pCambia1300. On the same vector dsRed under the control of a napin promoter was inserted, to allow for visible selection of transgenic seeds. Cloning and amplification of the plasmid was done in *E.coli* DH5α. For transformation of *Arabidopsis*, *Agrobacterium tumefaciens* strain C58C1 was used. To obtain the construct that was used for producing *35S::RBRi* seedlings the first 1000bp of the *RBR* genomic sequence was fused to 400bp from the corresponding cDNA. For selection, also the visible *NAPIN::DSRED* marker was used. For production *RBRcs* seedlings the complete sequence upstream of *RBR* (until the transcription start of the next gene) and the complete sequence downstream of *RBR* (until transcription start of the next gene) was fused to the complete coding sequence of *RBR* into the binary vector pCambia1300. *RBR::GUS* plants were constructed by cloning the upstream promoter-region of *RBR* to the *GUS* gene in pCambia1300. For transformation of *Arabidopsis* (Columbia accession) the floral dip method was used. T1 seeds were either selected via fluorescence of the seed coat or on Murashige and Skoog (MS) medium containing 50µg/ml kanamycin and after around two weeks the seedlings were transferred to soil. Subsequent generations were grown in Conviron growth chambers (mixed fluorescent an incandescent light 230µmol/m2/s at 22°) under long day condition (16h light). For all experiments seeds were sterilized according to standard methods, and stratified for 4 nights at 4° in the dark on growth medium, which consisted of MS medium supplemented with either 1% or 3% sucrose or equivalent-molar amounts of glucose, glucose-6-P or mannitol as control.

Protein gel blot analysis

Protein extracts were prepared from *Arabidopsis* by grinding shock-frozen tissue. Subsequently extraction buffer was added (7M urea, 2M thiourea, 10% v/v isopropanol, 5% v/v glycerol, 2% v/v pharmalyte, 50mM DTT, 1xComplete protease inhibitor cocktail (Roche)). Homogenates were centrifuged 2x20min at RT. Protein concentration was equilibrated (using a simple bradford method with the Roth-Nanoquant-solution according to manufacturers protocol) until all samples contained the same concentration. Laemmli-buffer was added and 100µg of protein was added to each lane of an 8% SDS PAGE. For each Western-blot in parallel as loading control another gel was prepared which was subsequently coomassie stained according to standard procedures. Blotting was performed semi-dry onto nitrocellulose in 20% v/v MeOH, 0.29% w/v

glycine, 0.58% w/v Tris-base, 0.04% w/v SDS at 0.2V/cm2 for 2h. The membrane was incubated over night at 4° in TBST (150mM NaCl, 50mM Tris pH 7.5, 0.1% Tween20) with 5% w/v dry milk powder. Blots were subsequently incubated for 3h with a 1:400 dilution of α-RBR antibody in TBST plus milk. After 3x10min washing in TBST secondary α-rabbit antibody 1:5000 in TBST plus milk was added and the blot was incubated for another 2h. After 4x final washing chemiluminescent detection ws performed with the ECL-enhancer kit from Bio-rad according to manufacturer`s instructions.

Antibody production

For antibody production the N-terminal domain of RBR (encoding the first 374 amino acids) were cloned into the expression vector pQE31 which offers an N-terminal 6Xhistidine tag and transformed into *E.coli* M15Rep4 cells. Subsequently primary transformants were screened for high expression of the protein with a colony-blot procedure according to instructions from the Quiagen-expressionist (http://www1.qiagen.com/literature/handbooks/PDF/Protein/Expression/QXP_QIAexpressionist/1024473_QXPHB_0603.pdf). Induction and purification was done under native conditions as described in the quiagen-expressionist. In short: a preculture was grown over night in LB-medium at 30° in 200mg/l AMP and 50mg/l kan; next day transfer 1:100 in SB (35g tryptone, 20g yeast, 5g NaCl/l) without AB @ 28° - until o.d. (optical density) has reached 0.4; shift to 18° and adaptation for 1h; induction with 500µM IPTG; harvest of cells when o.d of 1.5 has been reached. Harvest/lysis (at 4°): 15min @ 10000rpm (SLA 3000 rotor) resuspend in 2.5ml (50mM Na-Phosphate, 300mM NaCl, 10mM Imidazol)/100ml culture – and centrifugation for 12000rpm 5min (SS34 rotor), discharge supernatant, resuspend again in 2.5ml/100ml lysis buffer (50mM Na-Phosphate, 300mM NaCl, 10mM Imidazol, 4µg lysozyme); incubation 1h and sonification. Centrifugation (SS34) 20000 rpm for 1h;

For purification 200µl of Talon-rasin-beads per 100ml of culture was used. Further purification according to the quiagen expressionist. Instead of sodium-phosphate buffer, for elution 0.1Mops-buffer p.H. 7.7@4° was used. Protein was dialysed in mops-buffer and used for immunization of rabbits (3x) or coupled to the affi-gel15 (Bio-rad) raisin according to manufactures protocol. Subsequently 5ml of Serum was 10X diluted in TBS and passed over a column (N-terminal RBR coupled to 1ml affi-gel15) 5 times. After washing, antibodies were eluted 10x with 500µl 0.1 glycine and collected in Eppendorf reaction tubes with 42µl 1M Tris. Purity of antibodies was confirmed on dot-and Western-blots.

RNA isolation and Q-PCR

RNA was extracted using Trizol (Invitrogene) according to manufacturers instructions. For Q-PCR, RNA was treated with DNase I. 2µg of RNA was reverse-transcirbed using oligo(dT) primers and superscript (Invitrogene). Aliquots of the generated cDNA were used as template for PCR with gene specific primers (table material and methods). Q-PCR was performed in an ABI Prism 7700 Sequence Detection system (Applied biosystems AB), using FAST SYBR Green Master Mix reagent (AB) according to the manufacturer's instructions. All amplification plots were analyzed with a fluorescent signal threshold of at least 0.1 to obtain Cycle Treshold values. Experiments were performed in duplicate with error bars representing the range. Gene expression levels were normalized to pp2a as control gene, which was the most stable gene in seedlings according to the Biomarker discovery tool from Genevestigator (Zimmermann et al. 2004).

Microarray hybridization and evaluation

RNA was extracted from 3d old seedlings. The experiment was performed with 3 biological replicas. Affymetrix *Arabidopsis* ATH1 GeneChips were used in the experiment. Labeling of samples, hybridizations and measurements were performed as described in (Hennig et al. 2004). Signal values were derived using GCRMA algorithm in the statistical package R. Significance of differential 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 fold change at least 2. Significance of overlaps of gene-sets were calculated with an hypergeometric distribution and R. PCA analysis was performed with MEV (http://www.tm4.org/), using the full algorithm and standard settings. Clusteranalysis was done with the Genevestigator tool (https://www.genevestigator.com/gv/index.jsp).

Protein detection with mass-spectrometry

Equal amounts of 3d old shock-frozen seedlings were grinded and dissolved in extraction buffer (50mM Tris-HCL pH 6.8, 50mM NaCl, 4% SDS, 5% v/v Glycerol). Aliquots were diluted and protein concentration was determined using a BCA Protein Assay Kit (Thermo scientific). 50µgProteins/lane were subjected to SDS PAGE on 12% gels. After electrophoretic separation of the proteins, the gels were cut into 10 pieces for each fraction. Each gel slice was diced into small pieces. In gel digestion was performed according to (Shevchenko and Shevchenko 2001). Mass spectrometry measurements were performed on an LTQ FT-ICR (Thermo Finnigan), coupled with a Probot (LC-

Packings/Dionex) autosampler system and the UltiMate HPLC-system (LC-Packings/Dionex). Peptide mixtures were loaded onto laboratory made capillary columns (75 µm inner diameter, 8 cm length, packed with Magic C18 AQ beads, 3 µm, 100 Å (Microm)). Peptides were eluted from the column by an increased acetonitrile concentration in the mobile phase from 5% acetonitrile, 0.2% formic acid to 40% acetonitrile, 0.2% formic acid over 75 minutes, followed by a 10 minute wash step at 5% acetonitrile, 0.2% formic acid. Peptide ions were detected in a survey scan from 300 to 1'600 amu followed by 3 data-dependent MS/MS scans (isolation width 2 amu, relative collision energy 35%, dynamic exclusion enabled, repeat count 1, followed by peak exclusion for 2 minutes).
Interpretation of MS/MS spectra and data filtering MS/MS spectra were searched with TurboSequest and PeptideProphet by using the Trans-Proteomic Pipeline (TPP v2.9) against the *Arabidopsis thaliana* TAIR8 protein database (download on December 14th 2007) supplemented with contaminants. The search parameters were: requirement for tryptic ends, one missed cleavage allowed, mass tolerance = -/+ 3 Da, variable modification of methionine (M, PSI-MOD name: oxidation, ModAccession: MOD:00412, mono Δ = 15.9949) and static modification of cysteine (C, PSI-MOD name: iodoacetamide derivative, ModAccession: MOD:00397, mono Δ = 57.021464).
For PeptideProphet, the cutoff was set to a minimum probability of 0.9. APEX factors were determined according to (Lu et al. 2007).

Histological and Cytological analysis
Tissue was fixed in ethanol:acetic acid (9:1) dapi-stained and observed with a Zeiss Axioplan microscope. For anilin staining tissue was fixed (ethanol: acetic acid 7:1) over night. Afterwards tissue was submerged in 100% ethanol, washed with phosphate buffer (100mM sodium phosphate pH 9). Afterwards it was washed again and put on ice. Then, phosphate buffer was replaced with anilin blue staining solution (100mM sodium phosphate pH 9, 0.02% w/v aniline blue). The wells containing tissue in staining solution were then sealed warped in aluminium foil put at 4° for two weeks and observed under a Zeiss Axioplan microscope.
GUS staining of transgenic plants was performed following a modified protocol from (Sieburth and Meyerowitz 1997). Plant tissues were treated with cold 90% v/v acetone, and incubated for 2h at 37°C in X-Gluc staining solution (50mM NaPo4, 5mM K3Fe(CN)6, 5mM K4Fe(CN)6, 5mM X-Gluc). Chlorophyll was removed using an ethanol series from 30% v/v to 100% v/v (each step 1h). Tissues were cleard with 50% to 100% v/v Roth-Istol (Roth) mounted on oil and observed with a Zeiss Axioplan microscope. For scanning electron microscope a CamScan CS-44 (emitter: LaB6, detector systems: EDX, Orientation Imaging Microscopy) was used together with provided software. Prints of the analyzed *Arabidopsis* tissues were obtained following the protocol from (Kwiatkowska 2004).

For fat-red staining Sudan Red 7B (MP Biomedicals), plant tissue was soaked in staining solution (50mg Fat Red 7B in 25ml PEG-300 incubated for 1h at 90°C – cooled down – equal volume of 90% glycerol added – left at room temperature) overnight and well rinsed with water before observation (according to (Tsukagoshi et al. 2007)). Areas of seedlings or nuclei were quantified with imageJ software.

Cytological analysis

For ploidy analysis tissue was cut into small pieces in 400µl extraction buffer (Partec, Münster, Germany), incubated for 20min on ice, filtered through a 30µM mesh, mixed with 1ml nuclear staining buffer (with DAPI from Partec) and after incubation on ice for 10min analysed with a Partec Ploidy Analyzer. For quantification the results of 2 independent preparations were averaged. Nuclear spreads were prepared according to (Dittmer et al. 2007). Staining was performed with 20µg/mL propidium iodide.

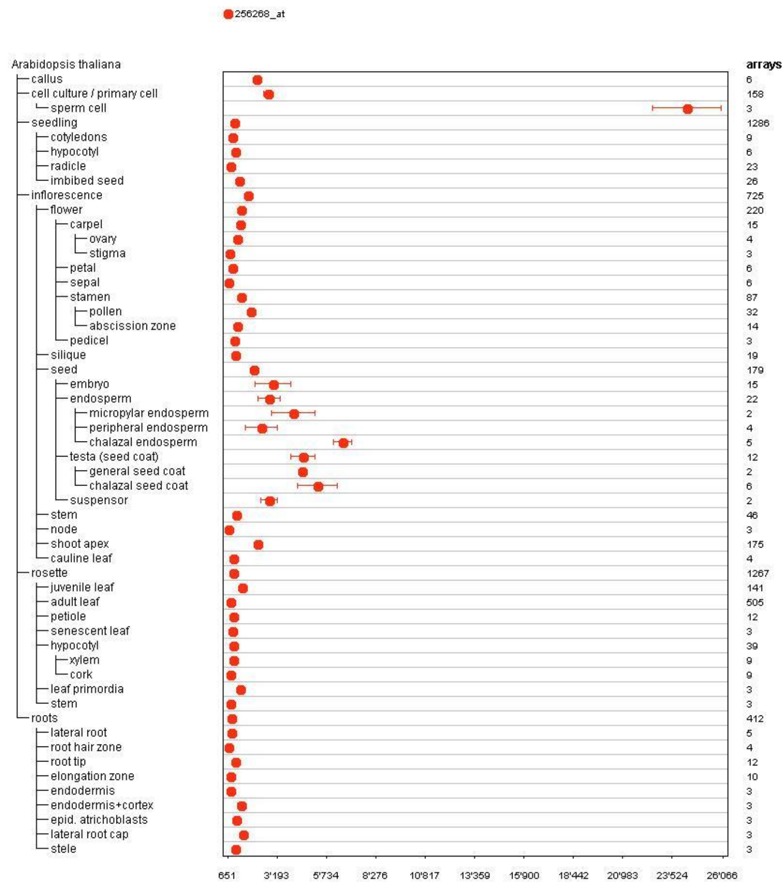
Fluorescent measurements

Flurescent measurements were performed with a Closed FC 800-C (Photon Systems Instruments) fluorcam with the provided software. Seedlings were arranged on plates two days before measurement. For measurement, seedling containing plantes were dark-adapted for 20min and the appropriate program from the soft-ware package was started.

Assay of sugar concentrations

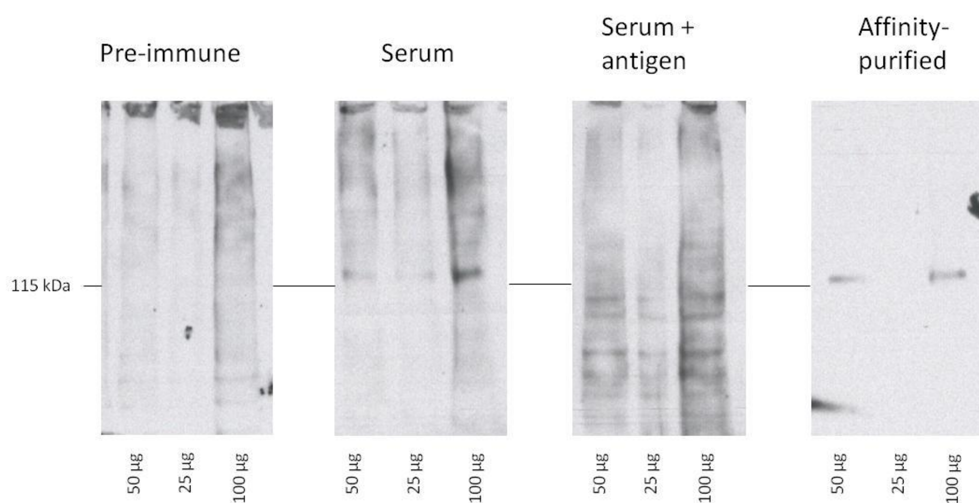
Equel amounts (around 20mg of fresh weight) of samples were powdered under liquid nitrogen, exactly weight out and were extracted by vigorous shaking with extraction buffer. Sucrose, glucose and fructose were determined in ethanolic extracts as in (von Schaewen et al. 1990). Starch was determined as in (Hendriks et al. 2003).

Supplementary information



Supplementary Figure 1. Expression of *RBR*.

The genevestigator anatomy-tool displayed a low, uniform expression of *RBR* in all tissues, except for the sperm cell were expression was highest. A slight increased expression is also visible for embryonic and seed tissue.



Supplementary Figure 2. Specificity of α-RBR. Antibodies were raised in rabbit against the N-terminal domain of RBR. In the serum a band at around 115kDa was visible that was not present in the pre-immune. The band disappeared when the blotting membrane was co-incubated with the antigene. All experiments were done with affinity-purified antibodies (using the antigen).

ID	Generation	# of Seeds	total # of pants	# of RBR-CS	Ration in %
RBRcs1	T2	363	257	59	23%
	T3	207	194	40	21%
	T3	301	284	117	41%
	T3	237	237	104	44%
RBRcs2	T2	162	162	47	29%
RBRcs3	T2	93	85	30	35%
RBRcs4-8	T2				<10%

Supplementary Table1. Segregation of 8 different *RBRcs* lines.

Supplementary Figure 3. *RBRcs* seedlings grown on different sucrose concentrations.

Seedlings grown 14d on 0.01% sucrose were morphological indistinguishable from seedlings grown without sucrose. Seedlings grown on 0.1% sucrose showed first morphological alterations.

probeset	agi	annotation	fold change	p-value
260716_at	AT1G48130	ATPER1 (Arabidopsis thaliana 1-cysteine peroxiredoxin 1); antioxidant	490	5.53E-86
249353_at	AT5G40420	OLEO2 (OLEOSIN 2)	408	1.37E-108
266544_at	AT2G35300	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein	307	1.70E-49
246299_at	AT3G51810	ATEM1 (Early methionine labelled)	264	4.30E-32
258224_at	AT3G15670	late embryogenesis abundant protein, putative / LEA protein, putative	247	1.58E-19
245335_at	AT4G16160	ATOEP16-2/ATOEP16-S; P-P-bond-hydrolysis-driven protein transmembrane transporter	232	1.07E-98
259167_at	AT3G01570	glycine-rich protein / oleosin	203	2.78E-146
258347_at	AT3G17520	late embryogenesis abundant domain-containing protein / LEA domain-containing protein	160	2.70E-25
250648_at	AT5G06760	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein	156	5.30E-17
247437_at	AT5G62490	ATHVA22B (Arabidopsis thaliana HVA22 homologue B)	155	3.02E-23
249082_at	AT5G44120	CRA1 (CRUCIFERINA); nutrient reservoir	152	3.60E-15
252019_at	AT3G53040	late embryogenesis abundant protein, putative / LEA protein, putative	111	3.94E-48
250624_at	AT5G07330	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G63060.1); similar to unknown [Xerophyta humilis] (GB:AAT45004.1)	109	6.66E-40
264079_at	AT2G28490	cupin family protein	81	1.13E-64
249772_at	AT5G24130	similar to unnamed protein product [Vitis vinifera] (GB:CAO69379.1)	75	1.32E-80
263789_at	AT2G24560	carboxylesterase	71	5.07E-08
264494_at	AT1G27461	similar to unnamed protein product [Vitis vinifera] (GB:CAO61483.1)	64	3.12E-27
263753_at	AT2G21490	LEA (DEHYDRIN LEA)	63	4.77E-38
256354_at	AT1G54870	oxidoreductase	61	9.17E-24
255905_at	AT1G17810	BETA-TIP (BETA-TONOPLAST INTRINSIC PROTEIN); water channel	58	1.54E-18
266654_at	AT2G25890	glycine-rich protein / oleosin	56	2.49E-19
265211_at	AT2G36640	ATECP63 (EMBRYONIC CELL PROTEIN 63)	51	1.16E-27
247095_at	AT5G66400	RAB18 (RESPONSIVE TO ABA 18)	50	1.02E-32
256442_at	AT3G10930	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G05300.1)	48	1.29E-16
257234_at	AT3G14880	similar to DOG1 (DELAY OF GERMINATION 1) [Arabidopsis thaliana] (TAIR:AT5G45830.1); similar to tumor-related protein-like, putative [Medicago truncatula] (GB:ABD32966.1)	47	1.45E-45
254440_at	AT4G21020	late embryogenesis abundant domain-containing protein / LEA domain-containing protein	37	3.91E-27
249039_at	AT5G44310	late embryogenesis abundant domain-containing protein / LEA domain-containing protein	35	8.00E-23
256416_at	AT3G11050	ATFER2 (FERRITIN 2); ferric iron binding	33	1.18E-24
250501_at	AT5G09640	SNG2 (SINAPOYLGLUCOSE ACCUMULATOR 2); serine carboxypeptidase	32	6.58E-28
264469_at	AT1G67100	LBD40 (LOB DOMAIN-CONTAINING PROTEIN 40)	31	3.68E-18
253767_at	AT4G28520	CRU3 (CRUCIFERIN 3); nutrient reservoir	31	5.02E-09
267317_at	AT2G34700	pollen Ole e 1 allergen and extensin family protein	31	9.24E-21
258498_at	AT3G02480	ABA-responsive protein-related	30	1.86E-11
266462_at	AT2G47770	benzodiazepine receptor-related	30	1.47E-09
259217_at	AT3G03620	MATE efflux family protein	29	9.92E-59
249937_at	AT5G22470	NAD+ ADP-ribosyltransferase	28	8.27E-41
254203_at	AT4G24150	AtGRF8 (GROWTH-REGULATING FACTOR 8)	28	2.21E-42
256898_at	AT3G24650	ABI3 (ABA INSENSITIVE 3); DNA binding / transcription activator/ transcription factor	27	4.02E-35
263907_at	AT2G36270	ABI5 (ABA INSENSITIVE 5); DNA binding / transcription activator/ transcription factor	26	7.64E-27
264400_at	AT1G61800	GPT2 (glucose-6-phosphate/phosphate translocator 2); antiporter/ glucose-6-phosphate transmembrane transporter	26	1.78E-33

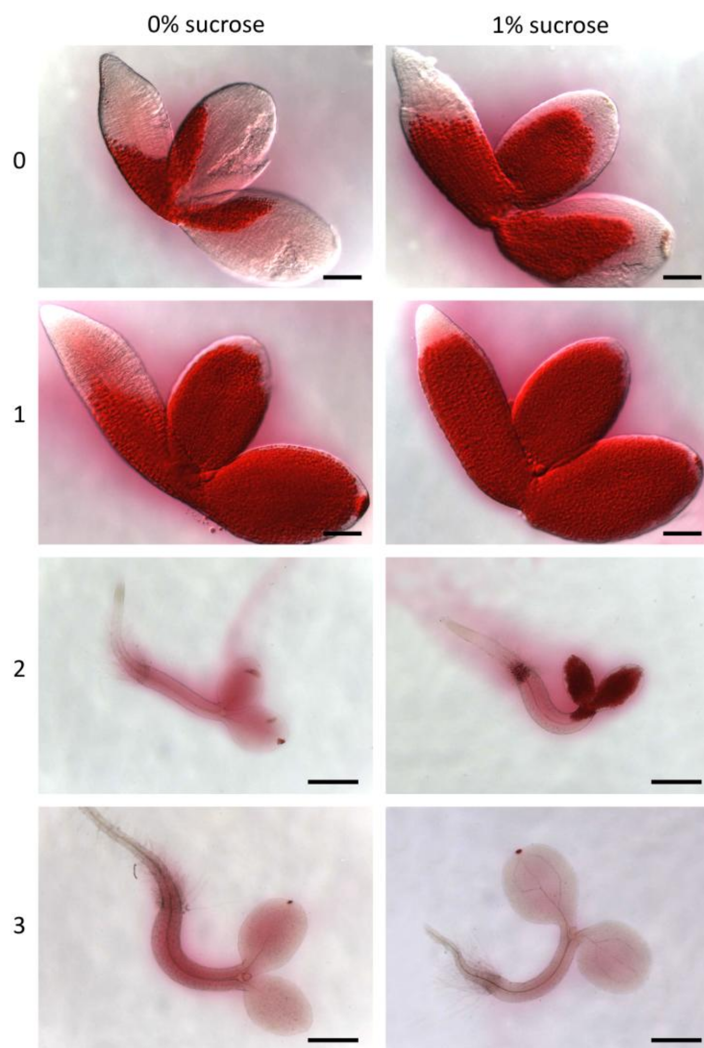
Supplementary Table2. Embryo-specific genes upregulated in *RBRcs*.

From 100 genes with strongest increase in expression compared to wild type in *RBRcs* grown on medium containing 1% sucrose, 40 embryo-specific genes were not deregulated in *RBRcs* seedlings grown without sucrose. Also these genes were not induced by sucrose in Col-0 seedlings.

ATG	annotation	change (log)
at5g44120	CRA1 (CRUCIFERINA); nutrient reservoir	7.2
at5g06760	late embryogenesis abundant group	7.3
at4g28520	CRU3 (CRUCIFERIN 3); nutrient reservoir	5.0
at1g54870	oxidoreductase	5.9
at3g15670	late embryogenesis abundant protein	7.9
at3g02480	ABA-responsive protein-related	4.9
at3g01570	glycine-rich protein / oleosin	7.7

Supplementary Table3.

Genes induced by *ABI3* overexpression and strongly induced in *RBRcs* grown on medium containing 1% sucrose.



Supplementary Figure 4. Lipid staining after germination.

Seedlings were stained with sudan-red, 0h, 1d, 2d and 3d after end of imbibitions (4d was always zero staining). Conspicious areas of the seedling were occupied by oil-bodies. This area was measured and related to the overall area of the seedling.

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4. The *Arabidopsis* RBR interactome reveals novel functions of the retinoblastoma pathway

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Abstract

Retinoblastoma related proteins (RBRs) regulates a variety of cellular processes. However, the molecular mechanisms that underlie these functions are in many cases still unclear. In order to decipher the molecular actions that govern *Arabidopsis* RBR function in plant cell cycle and development we used a co-immunoprecipitation approach to identify RBR associated protein complexes. With subsequent tandem mass spectrometry and data analysis we were able to suggest a protein interaction network around RBR. Using expression data of plants with reduced levels of RBR we could confirm the biological significance of some of the interactions. We were also able to accurately map three phosphorylation sites of RBR and two methylation sites, which comprise a new post-translational modification of retinoblastoma proteins.

Introduction

Animal retinoblastoma protein (pRB) is involved in many cellular responses, including differentiation of certain cell types, cell-cycle arrest and apoptosis. The best understood molecular interaction of pRB is its interaction with E2F transcription factors. E2Fs form complexes with their dimerizing partners DP and activate the expression of genes with E2F sites in their promoters, mainly genes required for the entry into S-phase (Burkhart and Sage 2008; van den Heuvel and Dyson 2008). Association of pRB with E2F/DP leads to repression of these genes. When pRB is phosphorylated by cyclin-dependent kinases, it cannot bind E2F anymore, thus allowing cells to progress into S-phase. Conversely, upon DNA damage phosphorylated pRB can bind to E2F in an activating complex, which induces expression of pro-apoptotic genes (Ianari et al. 2009). Moreover, DNA damage triggers histone acetyltransferase p300 to acetylate E2F1, and only acetylated E2F1 associates with proapoptotic promoters (Pediconi et al. 2003; Ianari et al. 2004). This has the interesting implication that only dividing cells undergo enhanced apoptosis and that E2F1 together with pRB participate in a proapoptotic pathway independent of p53 (Pediconi et al. 2003; Ianari et al. 2004).

Besides its interaction with E2F transcription factors, pRB has been demonstrated to physically interact with many other proteins, for example with the anaphase promoting complex APC (Binne et al. 2007), with histone deacetylase1, HDAC1 (Brehm et al. 1998; Luo et al. 1998), cyclinD (Dowdy et al. 1993; Kato et al. 1993), histone methyltransferase 1 (Robertson et al. 2000), ATP-dependent helicase BRG1 (Dunaief et al. 1994), replication factor C (Pennaneach et al. 2001), viral oncoproteins E1A and E7 (Hu et al. 1990), and transcription factors ID2 and EID-1, which promote dedifferentiation (Iavarone et al. 2004; Hassler et al. 2007). In 2001 a review was published describing more than 100 proteins that could bind pRB (Morris and Dyson 2001), however the biological meaning is in many cases not clear and most of these interactions still await confirmation.

In plants and animals, the core cell cycle machinery is conserved; therefore plants contain homologs of pRB, retinoblastoma-related proteins (RBRs), E2F/DPs and CDK complexes. Increasing evidence suggests that plants also use the retinoblastoma pathway to control the G1/S phase transition (Durfee et al. 2000; Gordon-Kamm et al. 2002; Park et al. 2005).

Very little is known about the molecular mechanisms that mediate RBR functions in plants. Proteins that have been found binding to RBR in plants are mostly cell cycle regulatory and viral proteins. Association of RBR with E2FA, E2FC cyclinD, Msi1 and several viral proteins has been demonstrated (Ach et al. 1997a; Ach et al. 1997b; del Pozo et al. 2002; Magyar et al. 2005; Desvoyes et al. 2006; Lageix et al. 2007; Jullien et

al. 2008). However almost nothing is known from plant RBRs about associations with proteins different from cell cycle regulators and how molecular interactions of RBRs are integrated into developmental processes. These questions are also very interesting in an evolutionary perspective, since plants and animals diverged before becoming multicellular and display very different modes of differentiation and development.

A number of functionally important post-translational modifications have been detected in retinoblastoma proteins. Animal pRB has 16 predicted phophorylation sites and many have been verified experimentally (Adams 2001). During cell cycle progression pRB phosphorylation is mediated sequentially by a series of CDK complexes (Adams 2001). Plant RBRs contain a similar number of predicted phosphorylation sites (Durfee et al. 2000) and it has been shown that hyper- and hypophorphorylated RBR populations resides in certain tissues (Shimizu-Sato et al. 2008).

Other post-translational modifications occuring in animal pRBs include acetylations close to the C-terminus (Chan et al., 2001; Nguyen et al., 2004), sumoylation (Ledl et al. 2005) and caspase cleavage (Borges et al. 2005). Acetylation of pRB seems to have a role in preventing phosphorylation (Chan et al. 2001) and promoting cell differentiation (Nguyen et al. 2004). The significance of sumoylation is not clearly understood yet and caspase-cleavage seems to be important to enter the apoptotic pathway (Borges et al. 2005).

To date in plants, no experimental data are available on the exact nature of any of these post-transcriptional modifications of RBRs. Such information might constitute an important resource to decipher plant RBR function by using site-directed mutagenesis.

In this study we used a co-immuno-precipitation approach to detect potential new interactions of RBR with other cellular proteins. We identified 57 proteins associated with RBR and probably representing at least 6 different pathways or protein complexes. We could confirm several previously recognized interactions from plants and animals and defined new protein complexes that could potentially be involved in RBR function. Based on the proteins we found binding to pRB we propose a network of RBR interactions that consists of three modules: interactions to regulate core cell cycle processes, interactions that might mediate developmental and signalling pathways, and proteins that might be important to regulate stability and localization of RBR.

Additionally we were able to map accurately three phosphorylated residues on RBR and three methylated residues, a post-transcriptional modification that has not been recognized for retinoblastoma related protein before.

Results

A fraction of RBR persists in the cytoplasm

As a transcriptional modifier, RBR is usually considered to be active mainly in the nucleus. However, two studies have shown that RBR associates with proteins that are also present in the cytoplasm and that the nuclear import of RBR is depending on microtubule-integrity (Roth et al., 2007; Nakatani et al., 2005). To localize RBR in plant cells, we performed whole-mount immuno-localisation in root tips of 3 days old seedlings of a RBR over-expression line (*RBR-OE*), since it was difficult to obtain strong signals from wild-type Col-0 seedlings (Figure 1A). RBR-OE plants are phenotypically normal and we have no evidence for any developmental alteration caused by RBR overexpression. In non-dividing cells, a strong RBR signal was clearly detected in nuclei. In dividing cells that showed a mitotic figure, the RBR signal was less strong and distributed over the whole area of the cell, suggesting that a population of RBR remains stable during mitosis and might have a function in the cytoplasm.

Therefore, to identify new RBR binding proteins in co-immuno-precipitation experiments, we used whole-plant protein extracts rather than a fraction enriched for nuclear proteins.

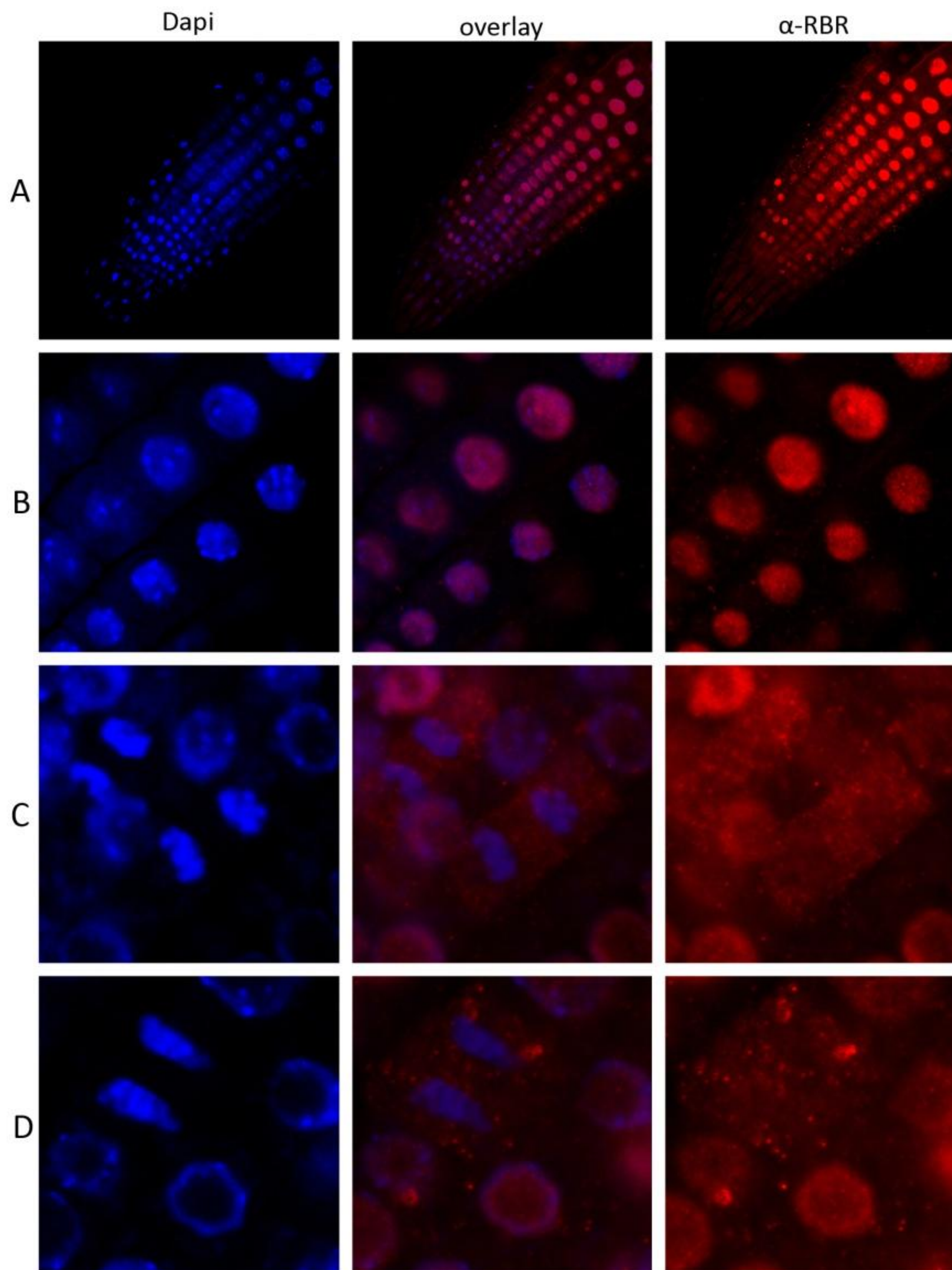

Figure 1: localisation of RBR. Immunolocalisation of RBR in root cells.

A: Overview of the root tip of 3d old *RBR-OE* seedlings showing mostly nuclear localisation of RBR. Nuclei were counter stained with DAPI.

B: close-up of A; RBR is mostly equally distributed within the nucleus. C

C and D: Cells in mitosis display mitotic figures. RBR was distributed all over the cytoplasm and was not degraded during mitosis.

RBR is present in multiple high molecular weight complexes.

The involvement of RBR in different pathways strongly suggests an interaction with many proteins within a cell. In order to find out whether RBR would associate to different protein complexes *in vivo,* we performed a gel filtration experiment of a crude protein extract from cell suspension culture. Cell suspension cultures, rather than complete plants offer an unlimited supply of protein extracts derived from dividing cells, expressing more than 85% of the core cell cycle regulators (Menges et al., 2005). *Arabidopsis* suspension cells contain relatively high levels of RBR (Figure 2B) and offer the possibility to grow large amounts of material in short time. The multiple fractions from the gel filtration step were analysed by immunoblotting, revealing the presence of RBR in high molecular weight complexes *in vivo* (Figure 2A). The RBR signal was distributed over size fractions spanning from more than1000kDa to around 100kDa, which is approximately the molecular weight of RBR. This suggested that RBR is either part of an unstable high molecular weight complex or is present in several different protein complexes of different sizes.

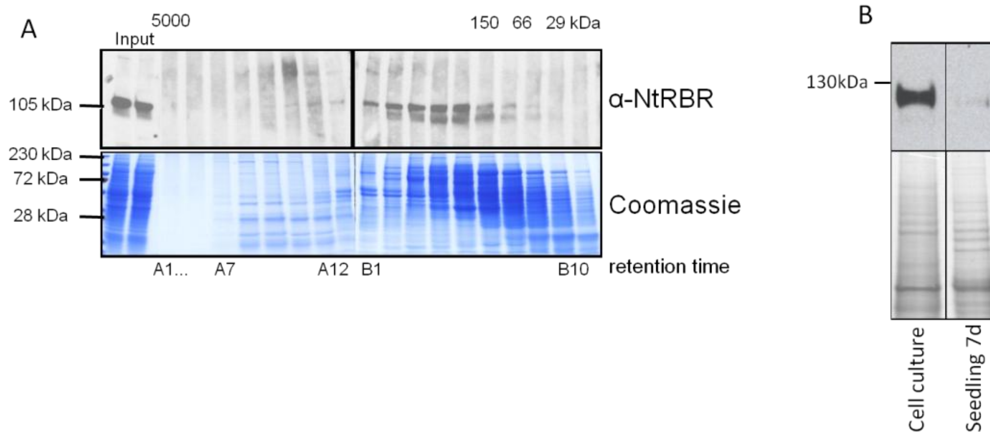

Figure 2:

A: A protein extract from cell suspension culture was separated by gel filtration. Western blotting showed a wide size distribution of RBR supporting the occurrence of RBR in high molecular weight complexes.

B: cell suspension cultures contain high levels of RBR protein compared to seedlings

Co-immunoprecipitation of RBR interaction partners

To characterize the composition of these protein complexes *in vivo*, we established a co-immunoprecipitation protocol for RBR with cell suspension culture (see material and methods for details). Using antibodies raised against the N-terminal domain of RBR (α-RBR), we could efficiently precipitate RBR (Figure 3A). A strong RBR signal on the western blot with little or no protein detected on the corresponding coomassie stained gel demonstrated the effective enrichment of RBR (Figure 3A). Increasing the protein input and amounts of α-RBR for precipitation enabled us to detect a coomassie stainable RBR band at 115kDa, which was not present in a mock-antibody control (Figure 3B). In control-experiments we used antibodies directed against an artificial storage protein (α-ASP1), which is not present in *Arabidopsis* (Zhang et al., 2003), since cell lines without functional RBR genes are not available.

After gel electrophoresis, co-immunoprecipitated proteins were subjected to tryptic digestion and analysed by ESI-MS/MS with a LTQ FT-ICR mass spectrometer. For robustness, we performed four biological replicas of this experiment.

RBR was detected in all four replicas with a total of 266 unique peptides (75, 62, 61 and 68 from the respective experiments), resulting in 53.2 % coverage of the sequence of RBR (Figure 4). In addition, we identified 204 other proteins in the precipitates. To identify the most reliable and specific potential RBR interaction partners from this list, we applied the following criteria: 1) proteins should not be detected in any of the α-ASP1-antibody control experiments; and 2) proteins should be identified in at least 3 of the four biological replicas or 3) in at least two replicas with a minimum of 6 unique peptides. 57 putative interaction partners remained, of which several (E2F/DPs from plants and animals; beta-tubulin, BIG, protein phosphatase and DNAJ from animals), were already known or proposed from previous work (Table 1). The remaining 43 proteins were identified as potentially new RBR-associated proteins. Based on their GO- and BLAST-annotations the 57 putative interaction partners were grouped into 8 functional groups (Table 1): transcriptional modifiers, protein translocators through the nuclear pore, proteins involved in DNA metabolism, chaperons, proteins involved in vesicular trafficking, members of the regulatory subunit of the 26S proteasome and a group of diverse proteins.

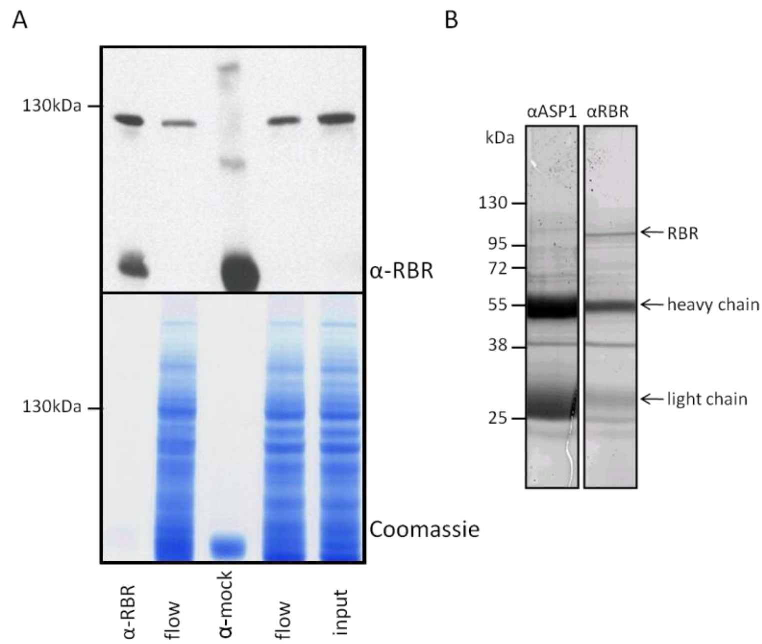

Figure 3: Co-immunprecipitation of RBR in cell suspension culture.

A: Efficient enrichment of RBR. The precipitated fraction was enriched in RBR and showed very little contamination as shown by coomassie-staine.

B: Co-immunoprecipated RBR was visible by coomassie staining after protein input and amount of antibody used was increased. As control, we used antibodies directed against an artificial storage protein that does not occur in Arabidopsis, but which was produced in the same animal (rabbit) as the RBR antibodies.

	protein	# peptides	description	L/IxCxE
Transcriptional modifiers	AT2G36010	10	E2FA	
	AT5G02470	10	DPA	
	AT3G12280	266	RBR	
	AT5G22220	31	E2FB	
	AT5G61070	6	HDA18	
	AT5G04640	12	AGL99	
	AT1G47870	7	E2FC	
	AT5G03415	16	DPB	
Cytoskeleton	AT5G44340	20	TUB4	
	AT4G20890	4	TUB9	
	AT5G12250	9	TUB6	
	AT1G64740	10	TUA1	LxCxE
	AT3G60740	4	TITAN 1	LxCxE
	AT4G14960	25	TUA6	LxCxE
Nuclear transport	AT2G16950	5	protein transporter	
	AT3G59020	9	protein transporter	LxCxE
	AT4G16143	7	importin alpha-2, putative	
	AT5G53480	9	importin beta-2, putative	
	AT5G06120	5	Ran-binding protein, putative	
Secretory pathway/ vesicle transport	AT1G26670	4	VESICAL TRANSPORT V-SNARE 12	
	AT2G27600	10	SKD1	
	AT4G31480	3	coatomer beta subunit, putative	
	AT3G02260	6	DOC1, BIG	IxCxE
	AT5G11980	6	Golgi complex component-related	LxCxE
	AT4G01400	10	PPR repeat-containing protein	LxCxE
	AT3G01780	8	TPLATE	
	AT5G16300	9	similar to hypothetical protein	LxCxE
	AT5G52340	4	exocyst subunit	
	AT2G38360	5	prenylated rab acceptor	
	AT3G56110	5	prenylated rab acceptor	
	AT2G40380	7	prenylated rab acceptor	
Nucleic acid/ DNA repair/ DNA synthesis	AT5G11170	3	DEAD/DEAH box helicase, putative	
	AT5G62190	6	plant RNA helicase 75	
	AT2G16440	4	DNA replication licensing factor, putative	
	AT5G22330	10	ATTIP49A	
	AT5G67630	3	DNA helicase, putative	IxCxE
	AT1G51690	4	protein phosphatase 2A 55 kDa	
Chaperones	AT3G44110	11	DnaJ homologue 3	
	AT5G22060	12	DnaJ homologue 2	
26S proteasome	AT1G53750	7	regulatory particle triple-A 1A	
	AT4G38630	0	REGULATORY PARTICLE NON-ATPASE 10	
	AT5G09900	4	RPN5A, EMBRYO DEFECTIVE 2107	
	AT1G20200	6	EMBRYO DEFECTIVE 2719	
	AT3G11270	4	RPN8A, ASYMMETRIC LEAVES ENHANCER 3	
	AT3G11910	12	ubiquitin-specific protease, putative	
	AT1G04810	4	26S proteasome regulatory subunit	
	AT2G20140	12	26S protease regulatory complex	
Enzymes; cell wall/ lipid metabolism	AT1G60440	4	ATPANK1, PANTOTHENATE KINASE 1	
	AT4G32180	48	ATPANK2, PANTOTHENATE KINASE 2	
	AT1G22410	9	2-dehydro-3-deoxyphosphoheptonate aldolase	
	AT3G29360	10	UDP-glucose 6-dehydrogenase, putative	
	AT5G16970	5	ALKENAL REDUCTASE	
	AT5G15490	3	UDP-glucose 6-dehydrogenase	
	AT2G16570	15	PYROPHOSPHATE AMIDOTRANSFERASE 1	
	AT2G36810	6	unknown protein	
	AT3G01010	3	UDP-glucose/GDP-mannose dehydrogenase	
	AT2G10920	4	unknown protein	
	AT3G54470	13	uridine 5'-monophosphate synthase	

Table1: RBR associated proteins grouped into functional categories. Also shown is the total number of peptides found in all four replicas and whether the protein contains a LxCxE or IxCxE RBR binding motiv.

Posttranslational modifications of RBR

To date, several posttranslational modifications have been reported for RBR in animals. With the strongly enriched RBR immunoprecipitate, we made use of the high mass accuracy of a LTQ FT-ICR mass spectrometer to detect posttranslational modification of RBR. Database searches with Mascot search engine and manual validation of the measured spectra revealed 3 phosphorylation sites and 3 methylation sites (Figure 4). In addition, we found an acetylation of the RBR N-terminus, but were not able to detect acetylation in the C-terminal region as was described for mammalian pRB (Chan et al., 2001; Nguyen et al., 2004).

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MEEVQPPVTPPIEPNGKRSEASLLDICEKVLSLDGSTCDEALKLFTETKRILS
ASMSNIGSGTREEVERFWFAFILYSVKRLSVRKEADGLSVSGDNEFNLCQILR
ALKLNIVDFFKELPQFVVKAGSVLGELYGADWENRLQAKEVQANFVHLSLLSK
YYKRGFREFFLTYDANAEKNSANSSTYLLDSYRFGWLLFLALRNHAFSRFKDL
VTCSNGVVSILAILIIHVPCRFRNFSIQDSSRFVKKGDKGVDLVASLCKIYDA
SEDELRIVIDKANNLVETILKKKPSPASECQTDKLDNIDPDGLTYFEDLLEET
SISTSLITLEKDYYDGKGELDERVFINEEDSLLGSGSLSAGAVNITGVKRKID
ALSSPARTFISPLSPHKSPAAKTNGISGATKLAATPVSTAMTTAKWLRTVISP
LLPKPSPGLEHFLKSCDRDITNDVTRRAHIILEAIFPNSSLGAQCGGGSLQAV
DLMDDIWAEQRRLEACKLYYRVLEAMCKAEAQILHANNLNSLLTNERFHRCML
ACSAELVLATHKTITMLFPAVLERTGITAFDLSKVIESFIRHEDSLPRELRRH
LNSLEERLLESMVWEKGSSMYNSLIVARPSLALEINQLGLLAEPMPSLDAIAA
LINFSDGANHASSVQKHETCPGQNGGIRSPKRLCTDYRSILVERNSFTSPVKD
RLLALGNVKSKMLPPPLQSAFASPTRPNPGGGGETCAETGINIFFTKINKLAA
VRINGMVERLQLSQQIRESVYCFFQHVLAQRTSLLFSRHIDQIILCCFYGVAK
ISQMSLTFREIIYNYRKQPQCKPLVFRSVYVDALQCRRQGRIGPDHVDIITFY
NEIFIPAVKPLLVELGPVRNDRAVEANNKPEGQCPGSPKVSVFPSVPDMSPKK
VSAVHNVYVSPLRGSKMDALISHSTKSYYACVGESTHAYQSPSKDLSAINNRL
NNSSSNRKRTLNFDAEAGMVSDSMVANSLNLQNQNQNQNGSDASSSGGAAPLK
TEPTDS*
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Acetylation
Phosphorylation
Methylation

Figure 4: Shown is the RBR sequence with posttranscriptional modifications. In red are sequences of peptides that were identified in all four replicas. N-terminal acetylation in green, phosporylated residues in red and methylated residues in blue.

Microarray analysis reveals functional overlap of RBR with E2Fa/DPa and importance of RBR for many cellular processes

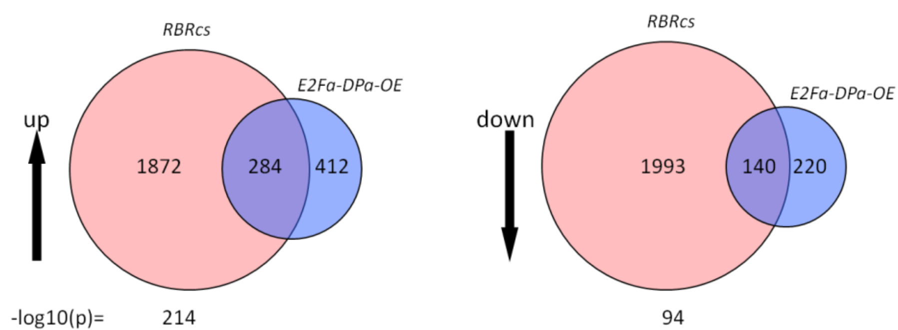
If RBR and E2Fa/DPa act in the same pathway, we hypothesized that there would be a significant overlap between genes affected in *RBRcs* and in *E2Fa/DP-OE* seedlings. In 3d old *RBRcs* seedlings 1872 genes were up and 1993 genes were down-regulated significantly at least two-fold. This was compared with gene expression changes in 6d old *E2Fa/DPa-OE* seedlings (Vandepoele et al. 2005). Around 2/3 of the genes that changed expression in *E2Fa/DPa-OE* seedlings were also deregulated in *RBRcs* seedlings (Figure 5). This strongly suggest that RBR and E2Fa/DPa act in the same pathway to regulate gene expression.

Next, as control for the biological significance of our interaction data we looked at specific gene expression changes in *RBRcs* seedlings. We reasoned that disturbance in certain cellular processes might lead to specific gene expression changes related to these processes. For example if RBR would have an important function during mitosis in the cytoplasm, loss of RBR might thus directly or indirectly lead to changes in expression of genes that are important during mitosis.

As potential interactors with RBR we found proteins related to nuclear transport, DNA damage response, vesicular transport, metabolic enzymes important for cell wall biosynthesis and lipid metabolism and the 26S proteasome. Therefore we calculated the enrichment of genes responsible for these processes using very specific annotations from mapman (Thimm et al. 2004). Figure 5 shows that the fraction of upregulated genes in *RBRcs* was strongly enrichment with genes for DNA replication, repair and cell division. Genes important for nuclear transport and cell organisation were slightly enriched. Genes for lipid metabolism were slightly enriched in both, up and down regulated genes. Genes important for cell wall synthesis were enriched in the fraction of downregulated genes. We found no enrichment of genes involved in proteasomal degradation or ubiquitination (Figure 5).

From this analysis we could confirms with certainty the importance of RBR only for DNA synthesis and cell division. However gene expression changes in *RBRcs* seedlings might not directly reflect defects in the RBR interaction network.

A



B

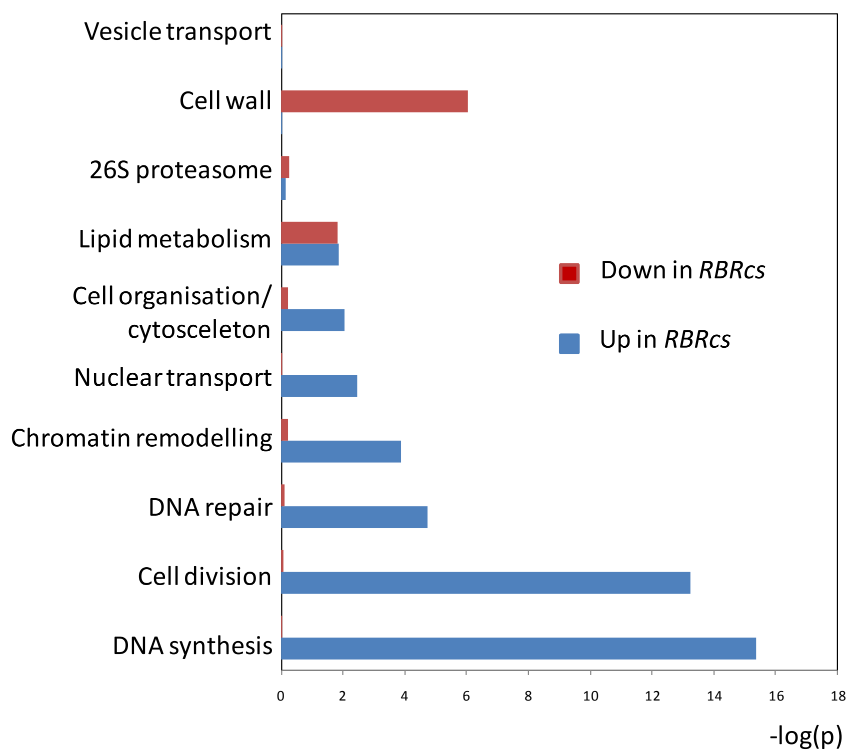


Figure5:

Comparing gene expression changes in *RBRcs* mutants and *E2Fa/DPa-OE* mutants revealed a significant overlap of deregulated genes. This suggest that RBR and E2Fa/DPa act in the same pathway. (Circles are not drawn to scale)

B: enrichment of genes that are present in the deregulated fraction of genes in *RBRcs* mutants. For annotation we chose bins from the map-man analysis tool.

Discussion

RBR localizion in the nucleus and in the cytoplasm

Whole-mount immuno-localisation in root tips and immunoblotting of isolated seedling nuclei showed that RBR strongly accumulated in the nucleus. However, during mitosis a stable fraction of RBR remained in the cytoplasm (Figure1). RBR could therefore be involved in the regulation of different cytoplasmic processes. Novel functions for RBR in the cytoplasm were supported by co-immunprecipitation of many cytoplasmic located interaction partners.

Established and novel RBR associated proteins in the nucleus

We found RBR associated with transcriptional modifiers E2Fa, E2Fb and E2Fc as well as with their dimerization partners DPa and DPb. For all three E2F transcription factors, the binding to DNA fragments carrying the canonical E2F site has been demonstrated (Mariconti et al., 2002). Also the binding of RBR to E2Fb and E2Fc *in vitro* has been shown (del Pozo et al., 2002; Magyar et al., 2005). However these experiments were performed either with *in vitro* transcribed and translated proteins or with heterologous expressed and purified proteins. Furthermore, to our knowledge binding of RBR to E2Fa has not yet been demonstrated. Overexpression of E2Fa together with its dimerization partner DPa led to ectopic cell division and increased endoreduplication (De Veylder et al., 2002). *RBRcs* seedlings also showed increased cell division activity but in contrast to the E2Fa/DPa overexpressor, a decrease in endoreduplication. When we compared the transcriptome of *RBRcs* seedlings with the transcriptome of seedlings with ectopic over-expressed E2Fa/DPa (Vandepoele et al., 2005) we found a highly significant correlation of genes with increased and decreased expression compared to wild type seedlings (Fig5). These results suggest strongly that RBR physically interacts with E2Fa, E2Fb, E2Fc and DPa and DPb. The phenotypic differences of *RBRcs* and *E2Fa-DPa-OE* with respect to endoreduplication indicate additional regulatory functions of RBR during the cell cycle other than controlling E2Fa/DPa activity.

Two other transcriptional modifiers, histone deacetylase HDA18 and a MADS-box containing protein AGL99, were associated with RBR by co-immunoprecipitation. AGL99, agamous-like 99 has been shown to interact with PHERES1 (de Folter et al., 2005) but has not been studied in more detail. Since MADS-box transcription factors occur only in plants, association of RBR and AGL99 would constitute a novel type of interaction. *hda18/-* mutants display defects in root epidermal cell patterning (Wang et al., 2005). Interestingly, the closest mammalian ortholog to *Arabdiopsis* HDA18 is HDAC6, a unique member of the histone deacetylase family, which deacetylates not only histones in the

nucleus (Grozinger et al., 1999) but also α-tubulin in the cytoplasm thus regulating microtubule dynamics (Hubbert et al., 2002). Furthermore HDAC6 has been implicated as a sensor for proteasome inhibition (Jiang et al., 2008) and until now it is the only HDAC member that has been shown to be required for efficient oncogenic transformation (Lee et al., 2008).

Furthermore we found 6 proteins involved in nucleic acid metabolism. Of these, ATTIP49a and its homolog At5g67630 are RUVB-like DNA helicases that could be important for DNA-recombination events but also for development. A T-DNA insertion mutant for *ATTIP49a* was embryo-lethal and an antisense line displayed strong meristem developmental phenotypes (Holt et al., 2002). Interestingly we also found an ortholog of human PP2A, a phosphatase which can modify the recruitment of pRB to chromatin in response to DNA-damage (Avni et al., 2003). The putative DNA-replication licensing factor (At2g16440) could indicate a role of RBR at the origin of replication.

In animals, E2F1 and pRB participate in an p53 independent pathway that leads to apoptosis (Ianari et al. 2009). Since the *Arabidopsis* genome does not contain p53, the master regulator that controls cell cycle decisions in response to DNA-damage, it might be interesting for future research to see if plant RBR together with E2Fs might have a role in regulating gene expression in resoponse to DNA damage in the plant cell.

Established and novel RBR associated proteins in the cytoplasm

We found RBR associated with several structural proteins including five different versions of tubulin and a tubulin folding cofactor, Titan1. In mammalian cells it has been demonstrated that the microtubule network is necessary for import of RBR into but not export out of the nucleus (Roth et al., 2007). Furthermore, it is tempting to speculate an involvement of HDA18 in this process. HDA18 shows more than 40% sequence identity with human histone deacetylase 6, HDAC6. As mentioned above HDAC6 is a regulator of microtubule dynamics in mammalian cells.

In addition, we found four proteins that belong to the nuclear-transport machine. One of these proteins (At4g16143, importin α-2) has been found in a previous study (Van Leene et al., 2007) as associated with the nuclear cyclin dependent kinase CDKD;2.

A number of probably cytosolic RBR interactors are involved in the secretory pathway. One of these is BIG; the mammalian ortholog of BIG, p600, has been described to interact with pRB (Nakatani et al., 2005). Nakatani et al suggested that pRB and BIG form a chromatin scaffold in the nucleus – however a functional interaction outside of the nucleus could not be excluded. *Arabidopsis* mutants for *BIG* are affected in vesicular trafficking, polar auxin transport, gibberelic acid signaling, light response and root

architecture (Gil et al., 2001; Kanyuka et al., 2003; Desgagne-Penix et al., 2005; Lopez-Bucio et al., 2005; Paciorek et al., 2005).

Other proteins of the secretory pathway that associated with RBR were coatproteins (At4g31480 for cargo recognition; At2g27600 part of the ESCRT complex), proteins for membrane fusion (prenylated rab acceptors: At2g38360, At3g56110 and At2g40380), one protein important for phragmoplast formation (At3g01780) and two other Golgi-associated proteins (At5g11980 and At5g16300).

The clear enrichment for proteins involved in vesicle trafficking indicated that RBR is involved in functions not directly connected to DNA based processes during the cell cycle, but rather to cell structure and signalling.

Two proteins that belong to the DnaJ group of chaperons have been found associated with RBR. This protein group shows sequence similarities to the large T-antigen of simian virus 40 (SV40), which is a known to target the human retinoblastoma-protein pRB (DeCaprio et al., 1988). The so-called J-domain of SV40 is important to functionally inactivate pRB (Sheng et al. 1997; Srinivasan et al. 1997; Stubdal et al. 1997; Zalvide et al. 1998). This could suggest that these chaperones are important for proper folding of RBR in the cytosol and have been structurally mimicried by SV40.

We also identified eight proteins involved in proteasomal degradation. 7 of these 8 are subunits of the regulatory, non-ATPase 19S-lid of the 26S proteasome. *Arabidopsis* mutants of these subunits showed pleiotropic effects on development, cytokinin signalling, glucose and abscisic acid signalling (Smalle et al., 2003; Jain et al., 2008; Book et al., 2009) (Cho et al., 2006). A weak defect in 26S proteasome activity via mutation of RPT2a led to enlarged cell and organ size (Kurepa et al., 2009) – in contrast to what has been described in mutants with reduced RBR activity (Park et al. 2005; Desvoyes et al. 2006; Lageix et al. 2007). This could be explained by regulation of the level of RBR by targeted protein degradation through the proteasome. Cytokinin has been shown to inhibit proteasome-mediated degradation (Jain et al., 2008), which could explain the accumulation of RBR in *RBRcs* seedlings when grown on medium supplemented with low concentrations of cytokinin (Suppl Fig1). The 19S lid of the 26S proteasome resembles structurally the cop9-signalosome and it has been proposed that they could substitute each other on the catalytic 20S subunit (Li and Deng, 2003). Components of the cop9 signalosome have first been identified as constitutive photomorphogenic mutants (Wei and Deng, 1992). The *Drosophila* homologs to RBR, RBF1 and RBF2 were protected from proteasomal degradation in cells with low expression of COP9 subunits (Ullah et al. 2007).

Several metabolic enzymes co-immunoprecipitated with RBR, including 3 of the 5 UDP-glucose dehydrogenases from the *Arabidopsis* genome. UDP-glucose is an important precursor for the synthesis of pectin, hemicellulose and callose (Hong et al., 2001;

Klinghammer and Tenhaken, 2007) thus playing an important role in cell expansion and cell plate formation. We also found both *Arabidopsis* pantothenate-kinases, Atpank1 and Atpank2. Apart from their central role in metabolism by generating the precursor of co-enzymeA, the Drosophila ortholog of Atpank2, fumble, localizes to the mitotic spindle and has been shown to be important for mitosis, meisosis and DNA integrity during Drosophila develoment (Afshar et al., 2001; Bosveld et al., 2008).

Posttranslational modifications of RBR

RBR contains several physiological important post-transcriptional modifications. Phosphorylation of animal pRB can determine whether it can participate in repressive complexes, inhibiting transcription of S-phase genes (Adams 2001) or whether pRB can participate in an activating complex inducing the expression of pro-apopctotic genes (Ianari et al. 2009). From plants only limited evidence is available that the activity of pRB is regulated by phosphorylation. The maize RBR1 protein undergoes changes in phosphorylation states concomitant with endoreduplication (Grafi et al. 1996) and association of maize RBR1 and tobacco NtRBR1 with CDK-complexes has been demonstrated (Huntley et al. 1998; Nakagami et al. 1999; Nakagami et al. 2002).

In axillary buds of pea the dormancy to growth transition correlates with a phosphoylation of pea RBR1 protein (Shimizu-Sato et al. 2008). We provide a mapping of three phosphorlylation sites of RBR. Furthermore we found phosphorylated and non-phosphorlylated peptides in the same size fraction. Consequently, besides hpyerphosphorylated versions of RBR which migrate differently on PAGE (Shimizu-Sato et al. 2008) there might exist oligo-phosphorylated and hypo-phophorylated versions of RBR. Mapping the phosphorylation sites gives the possiblitly to mutate RBR and to decipher specific functions of these sits. Additionally we found three methylated residues – a post transcriptional modification of RBR that has not been described before. It will be interesting to see whether this PTM can modify the function of RBR.

Construction of a RBR interaction network

Based on our findings and the previously known interaction partners of RBR, we speculated about an interaction network involving the different putative pathways regulated by RBR. We propose a network composed of three modules (Figure 6): The central, classical action of RBR is regulating the cell cycle via E2Fa, E2Fb, E2Fc and their dimerizing partners DPa and DPb. Additionally, RBR associates with proteins directly involved in replication control and might function in DNA-damage response. In the second module, RBR is mediating mitogenic stimuli to the cell cycle. This might be either via regulating proteins of the secretory pathway or compromising interactions with the

26S proteasome. The last module regulates RBR protein localization and stability. The activity of RBR could be controlled by regulating its localisation either in the cytoplasm or in the nucleus and this process might depend on HDA18, microtubules and importins. Also proteasomal degradation of RBR might play a role and could be modified by growth stimuli.

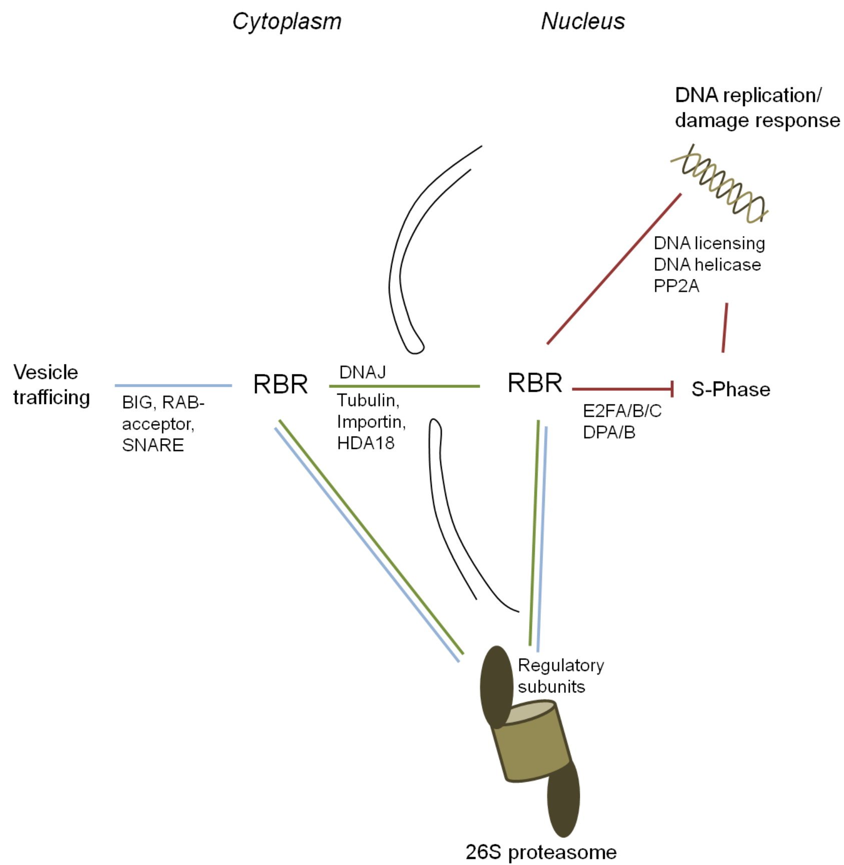


Figure6:

Hypothetical network of protein-interactions around RBR. Our data suggest that RBR is a mediator of at least 3 different modules. Connections in red show regulation of S-phase entry and DNA replication and DNA repair. In blue are interactions with proteins that might have implications for integration of developmental signals. Green depicts interactions around the life-cycle of RBR, protein folding, import into the nucleus and degredation via the 26S proteasome.

Conclusions

RBR is considered as central regulator of cell-fate decision and regulates a variety of cellular processes. In animals, many cellular functions have been shown to be linked to retinoblastoma proteins, however, the respective connections remain poorly understood in plants. In this work, we investigated the molecular interactions surrounding RBR. Co-immunoprecipitation coupled to tandem mass spectrometry revealed 57 putative interaction partners located in the nucleus as well as in the cytoplasm. Functionally, identified interactors mainly belonged to transcriptional modifiers, nuclear transport, DNA metabolism, chaperons, vesicular trafficking and to the regulatory subunit of the 26S proteasome. Finally, we speculate about an RBR interaction network to integrate the various pathways connected to RBR, to its life cycle regulation and to its putative activation via phosphorylation or methylation. The proposed RBR interaction network provides a basis for further research on the connection of cell cycle and development in *Arabidopsis*.

Material and Methods

Growth conditions

Arabidopsis thaliana Col-0 cells were cultured in MS medium containing 3% sucrose, 1× Murashagi & Scoog medium, 500 µg/L NAA, and 50 µg/L kinetin. One-fifth of the cell volume was transferred to fresh medium every 7 days. For preparation of protein extracts, cells were harvested after 7 days.

For production of *RBRcs* seedlings the complete sequence upstream of *RBR* (until the transcription start of the next gene) and the complete sequence downstream of *RBR* (until transcription start of the next gene) was fused to the complete coding sequence of *RBR* into the binary vector pCambia1300. For transformation of *Arabidopsis* (Columbia accession) the floral dip method was used. T1 seeds were either selected via fluorescence of the seed coat or on Murashige and Skoog (MS) medium containing 50µg/ml kanamycin and after around two weeks the seedlings were transferred to soil. Subsequent generations were grown in Conviron growth chambers (mixed fluorescent an incandescent light 230µmol/m2/s at 22°) under long day condition (16h light). For all experiments seeds were sterilized according to standard methods, and stratified for 4 nights at 4° in the dark on growth medium, which consisted of MS medium supplemented with either 1% or 3% sucrose or equivalent-molar amounts of glucose, glucose-6-P or mannitol as control.

Protein immunolocalisation

For whole mount immunolocalisation of RBR in root tips a published protocol was used (Paciorek et al. 2006).

Protein gel blot analysis

Protein extracts were prepared from *Arabidopsis* by grinding shock-frozen tissue. Subsequently extraction buffer was added (7M urea, 2M thiourea, 10% v/v isopropanol, 5% v/v glycerol, 2% v/v pharmalyte, 50mM DTT, 1xComplete protease inhibitor cocktail (Roche)). Homogenates were centrifuged 2x20min at RT. Protein concentration was equilibrated (using a simple bradford method with the Roth-Nanoquant-solution according to manufacturers protocol) until all samples contained the same concentration. Laemmli-buffer was added and 100µg of protein was added to each lane of an 8% SDS PAGE. For each Western-blot in parallel as loading control another gel was prepared which was subsequently coomassie stained according to standard procedures.

Blotting was performed semi-dry onto nitrocellulose in 20% v/v MeOH, 0.29% w/v glycine, 0.58% w/v Tris-base, 0.04% w/v SDS at 0.2V/cm2 for 2h. The membrane was incubated over night at 4° in TBST (150mM NaCl, 50mM Tris pH 7.5, 0.1% Tween20) with 5% w/v dry milk powder. Blots were subsequently incubated for 3h with a 1:400 dilution of α-RBR antibody in TBST plus milk. After 3x10min washing in TBST secondary α-rabbit antibody 1:5000 in TBST plus milk was added and the blot was incubated for another 2h. After 4x final washing chemiluminescent detection ws performed with the ECL-enhancer kit from Bio-rad according to manufacturer`s instructions.

Immunoprecipitation gel filtration and sample preparation

For each replica and each sample, 2 litres of cell culture material and 250µl of affinity-purified α-RBR antibody or 50µl of affinity-purified α-ASP1 antibody (Zhang et al. 2003). Cell culture material was filtrated and grinded under liquid nitrogen, then IP buffer (20mM HEPES/KOH pH 7.5 at RT, 0.1mM EDTA, 0.1M KCL, 10% glycerol, 0.5mM DTT, 0.02% Tween20, 2xCOMPLETE protease inhibitor cocktail (Roche)) was added and put at 4° until material was melted. Subsequent steps were all performed at 4°C. Protein concentration was equilibrated to 20mg/ml and extract was centrifuged two times (fist 20min SS34 10000rpm, second 20000rpm SS34 40min). Subsequently antibodies were added to the supernatant (50ml corresponding to 1g of protein per sample) and rocketed over night. For precipitation 200µl of washed Fast Flow ProteinA sepharose (Amersham) was added and rocketed for 4h. Next sepharose was washed extensively and eluted in 2XLaemmli buffer and samples were prepared for SDS-PAGE (12%) according to standard procedures. For gel-filtration protein extracts (300µl) were run on an Aekta explorer 900 (Amersham) with a superrose TM6 column (Code Number: 17-0537-01). 1,5ml fractions were collected and concentrated to 30µl with centriplus amicon centricons (0.8ml). PAGE and Western was performed as described.

After electrophoretic separation phosphorylated proteins were stained with colloidal coomassie (fixing in 12% TCA, staining over night: 20%MeOH, 12,5% AmmoniumSulfate w/v, 2,5% w/v Ortho-phophoric acid, 0.1% Coomassie G-250) and destained/washed several times in double distilled water. Gels were cut into 10 pieces for each fraction. Each gel slice was diced into small pieces. In gel digestion was performed according to a modified protocol from (Shevchenko and Shevchenko 2001). Before mass spectrometric analysis, samples got desalted using Sepak Cartridges (Waters, Milford, Massachusetts, USA).

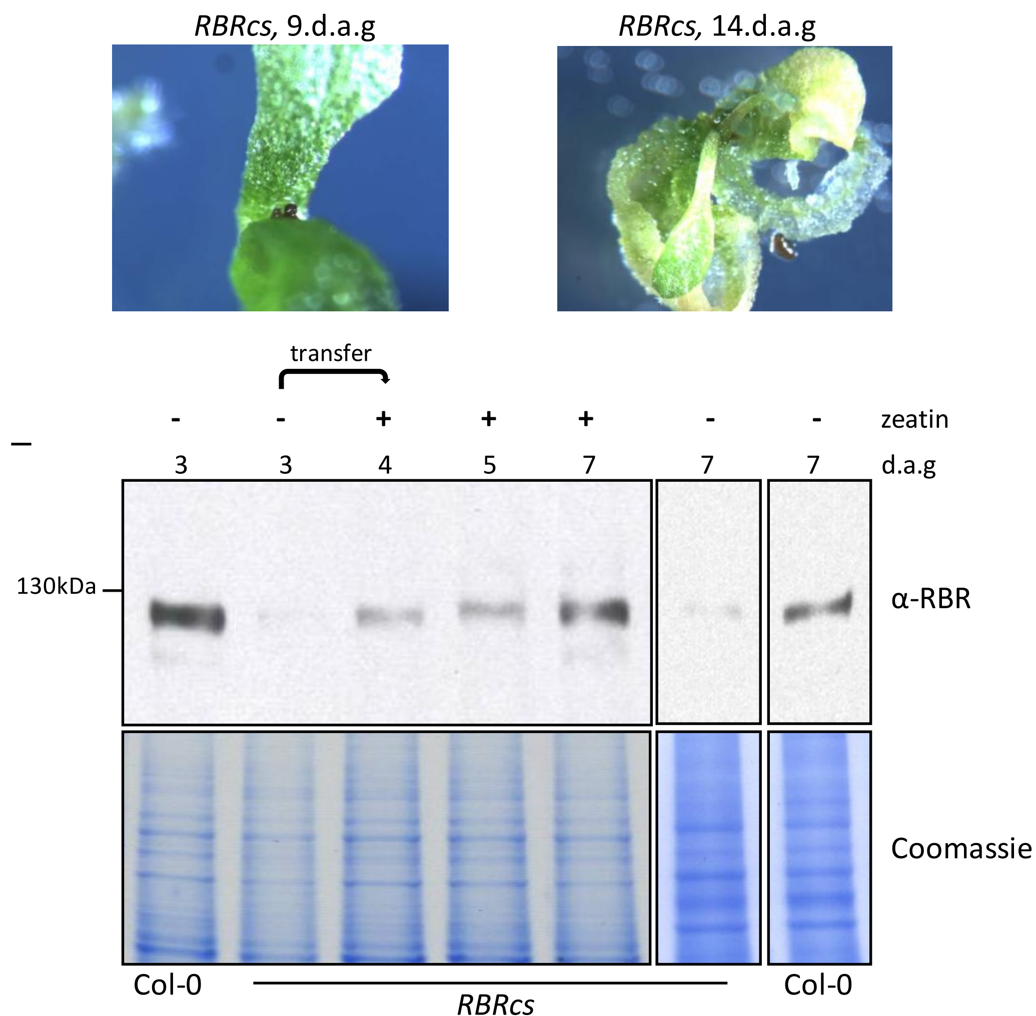
Analysis by LC-ESI-MS/MS

Dried peptides were resuspended in 3% ACN, 0.2% formic acid and analyzed on a LTQ FT-ICR Mass spectrometer (ThermoFischer Scientific, Bremen, Germany) coupled with a Eksigent nano LC system (Eksigent Technologies, Dublin, CA, USA). Peptide mixtures

were loaded onto laboratory made capillary columns (75 µm inner diameter (BGB Analytik, Böckten, Switzerland), 8 cm length, packed with Magic C18 AQ beads, 3 µm, 100 Å (Michrom BioResources, Auburn, CA, USA)). Peptides were eluted from the column by an increased acetonitrile concentration in the mobile phase from 5% acetonitrile, 0.2% formic acid to 40% acetonitrile, 0.2% formic acid over 74 minutes, followed by a 10 minute wash step at 5% acetonitrile, 0.2% formic acid. Peptide ions were detected in a survey scan from 300 to 1'600 amu at 100,000 FWHM nominal resolution followed by 3 data-dependent MS/MS scans (isolation width 2 amu, relative collision energy 35%, dynamic exclusion enabled, repeat count 1, followed by peak exclusion for 2 minutes).
Interpretation of MS/MS spectra and data filtering MS/MS spectra were searched with using Mascot 2.1.04 (Matrix Science, London, UK) against the *Arabidopsis thaliana* TAIR8 protein database (download on December 14th 2007) supplemented with contaminants. The search parameters were: requirement for tryptic ends, one missed cleavage allowed, mass tolerance = -/+ 3 Da. Beside carbamylation of cysteines as fixed modification, oxidation of methionine and phosphorylation of serine, threonine and tyrosine was included as variable modifications. Phosphopeptide identifications were accepted with a minimal MascotTM ion score of 30 and a MascotTM expect value of ≤ 0.01. A normalized delta ions-score (ΔI) was calculated for all phospho-, methyl- or acetylated peptides containing more than one putative modified residue by taking the difference of the two top ranking peptide ion scores and dividing that difference by the ion score of the first ranking peptide. Site assignments with a ΔI ≥ 0.4 were accepted.

Microarray hybridization and evaluation

The microarray experiment was already described before. Significance of overlaps of gene-sets were calculated with an hypergeometric distribution and R.



Supplementary figure1: Accumulation of RBR in response to cytokinin.

3d old *RBRcs* and Col-0 seedlings were transferred on medium containing 1μM of zeatin, and RBR levels were measured before transfer (3 d.a.g.) 1d, 2, and 3d after the transfer. RBR levels in *RBRcs* seedlings accumulated to wild type levels, which resulted in a partial recovery of the *RBRcs* phenotype (pictures on the top, seedlings were able to produce leaves, what never happened in *RBRcs* seedlings grown without cytokinin).

RBR levels did not change in response to zeatin in the wild type (not shown).

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5. *RBR* links transcriptional gene silencing, DNA repair and cell cycle in *Arabidopsis*

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Abstract

Studies from plant and animals demonstrated an important role of retinoblastoma-related (RBRs) proteins for cell cycle control, especially entry into DNA-synthesis phase. We used gene expression data of *Arabidopsis* seedlings with reduced levels of RBR (RBRcs) to mine for genes related to nucleic acid metabolism and DNA synthesis. Thereby we found genes required for biogenesis and function of short interfering RNAs and DNA *de novo* and maintenance methylation strongly upregulated. Conversely we found evidence for decreased activity of transgene silencing, *de novo* methylation and DNA repair. Furthermore mass-spectrometric quantification of proteins from wild type and *RBRcs* mutants suggested lower abundance of proteins from the transgene silencing pathway. We propose that the reduction of RBR protein in *Arabidopsis* leads to a decreased activity of transgene silencing and DNA *de novo* methylation and that RBR has an additional specific role in controlling these pathways on a posttranscriptional level.

Introduction

The retinoblastoma protein is an important regulator of the cell cycle and controls the G1/S phase transition. It is a direct inhibitor of E2F type transcription factors, which are necessary for the induction of S-phase genes. Growth stimuli result in an activation of cyclin dependent kinases which phosphorylate retinoblastoma proteins thus disabling them to bind to E2F (Elmayan et al. 2005; Burkhart and Sage 2008; van den Heuvel and Dyson 2008). This function makes retinoblastoma proteins very important tumor suppressors in mammalian cells. Retinoblastoma proteins are well conserved and are found in animals and plants. *Arabidopsis* mutants of the retinoblastoma related protein (RBR) are gametophytic lethal (Ebel et al. 2004) but several studies suggested that RBR regulates the cell cycle via E2F transcription factors also in plants (Park et al. 2005; Desvoyes et al. 2006; Fang et al. 2006; Lageix et al. 2007).

In this study we show that in *Arabidopsis* mutant seedlings with reduced levels of RBR (RBR cosuppression – *RBRcs*) a significant number of S-phase genes is activated. Also we found many genes involved in DNA maintaince *de novo* methylation and transcriptional gene silencing (TGS) upregulated. Genes for TGS and the *de novo* methylation are important for silencing of sequence repeats, viral genes and transposons.

In plants two pathways that result in *de novo* DNA methylation are known: RNA-directed DNA methylation (RdDM) and RNAi mediated heterochromatin formation (Matzke and Birchler 2005). For RdDM double stranded RNA is produced by RNA-dependent RNA polymerase 2 (RDR2) and processed by DCL3, which results in 21-24 nucleotides long small interfering RNAs. One strand of these is incorporated into the AGO4/6-RISC complex to target site-specific DNA sequences and the methyltransferses MET1 and DRM1 catalyse *de novo* DNA methylation. This DNA methylation can be maintained via the action of MET1, DDM1 and HDA6 for CG methylation or CMT3 and SUVH4 for CNG methylation.

RNAi induced heterochromatin formation has been shown to be important in *Arabidopsis* only for the formation of a heterochromatic knob on chromosome four (Lippman et al. 2004). However a lot more genes seem to be important for TGS (Elmayan et al. 2005) and future research will show their mode of action.

There is some evidence for a connection of retinoblastoma like proteins and DNA-*de novo* methylation/transcriptional gene silencing in animals. For example in *C. elegans* the retinoblastoma related mutant *lin-35* has been widely used for siRNA screens due to its more efficient silencing of target genes. A study from 2005 showed that in *C.elegans* mutations in RB pathway components enhance RNA interference and cause somatic cells to express genes and elaborate perinuclear structures normally limited to germline-specific P-granules (Borges et al. 2005). In *Arabidopsis* it has been demonstrated that

RBR controls imprinting in the female gametophyte via binding to the promoter of *MET1* (Jullien et al. 2008) but otherwise it is not known whether RBR plays also a role in *de novo* methylation. Here we show that AGO4, AGO6, DDM1, SUVH4, CMT3, DRM1, MET1, DCL3 and RDR2 display strongly elevated expression in *RBRcs* mutants. Therefore we predicted an increase in activity of the RdDM pathway similar to the situation in *C.elegans* were a mutation in *lin-35* results in an increase of TGS. In contrast, we found the opposite: multiple readouts of RdDM and TGS that we tested showed that these pathways had decreased or no activity. We also found evidence for a lower abundance of AGO4, AGO6 and DCL3 protein despite accumulation of mRNA. This reminded us about the situation of *RBR,* which also showed increased expression but decreased RBR activity and protein levels in *RBRcs* mutants. Therefore we speculate that the RBR pathway is connected to transcriptional gene silencing and *de novo* DNA methylation and likely acts not only on a transcriptional but also on a post-transcriptional level.

Genes of the DCL3 and DNA de novo methylation pathway show elevated expression in RBRcs mutants

RBRcs mutants are developmentally arrested after germination. Some of the growth defects can be rescued by sugar in the growth medium and *RBRcs* seedlings grown on medium with 1% sucrose resume cell division and produce short roots. We previously performed a microarray analysis of gene expression in 3d old *RBRcs* and wild type seedlings. Consistent with a role of RBR during G1/S-phase transition, we found a significant portion of genes related to nucleic acid metabolism strongly upregulated in *RBRcs* mutants (Supplementary figure 1, figure1 and 2A). When we mined the data more careful, we realized that almost all known genes of the *DCL3*, DNA *de novo* and maintainence methylation pathway showed increased expression in *RBRcs* seedlings (Table1). Many genes required for S-phase are regulated by E2Fa/DPa heterodimer transcription factors (De Veylder et al. 2002) and one of the important functions of RBR is to inhibit E2F activity. Therefore we looked whether we would find these genes also upregulated in E2Fa/DPa overexpressing (OE) seedlings. Most of them were significantly over-expressed in *E2Fa/DPa-OE* seedlings and furthermore contained typical E2F binding sites in their promoters (Table1). In order to confirm this elevated expression of TGS-and *de novo* DNA methylation pathway genes we measured their transcript levels with quantitative PCR. To detect a potential accumulation of the transcript we performed this experiment at two different time points and with or without sucrose. Figure2A shows that we always found increased expression of *SUVH4*, *DCL3*, *MET1*, *AGO4/6*, *DDM1* and *RPA2*.

In order to verify that RBR could bind to E2F-sites in the promoters of these genes, we performed a chromatin-immunoprecipitation experiment. The binding of RBR to the *MET1* promoter has already been shown (Jullien et al. 2008). We chose to focus on promoters of *DRM1* and *AGO6* because these genes were highly induced in *RBRcs* mutants. Due to its high content of RBR protein we isolated chromatin from exponentially growing *Arabidopsis* cell suspension culture as input material. Using antibodies against RBR we could detect an enrichment of DNA-fragments close to E2F sites of the promoters of *AGO6* but not of *DRM1* (not shown) in our precipitate (Figure1). As positive control we used the promoter region of *PCNA*, which has been shown to be targeted by RBR before (Hirano et al. 2008), and as negative control we used unspecific antibodies.

These experiments suggest that RBR is regulating the transcription of genes of the *DCL3* and DNA *de novo* methylation pathway via E2Fa/DPa. Consistent with a high accumulation of transcript we would expect to find an increased activity of these pathways.

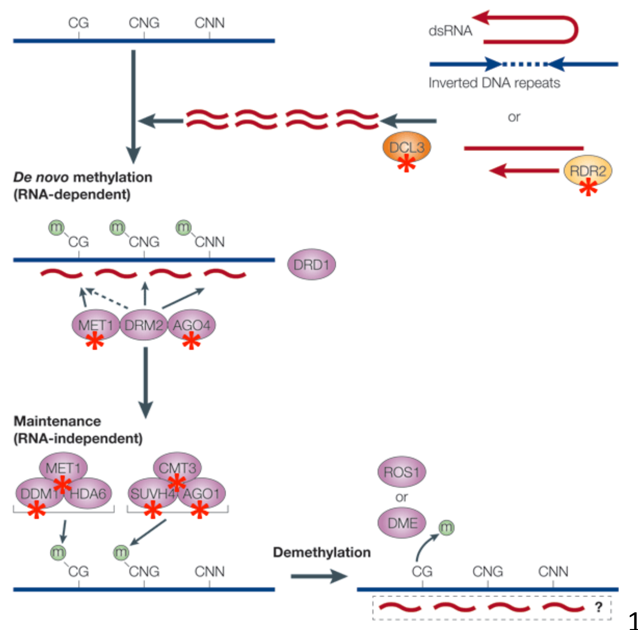
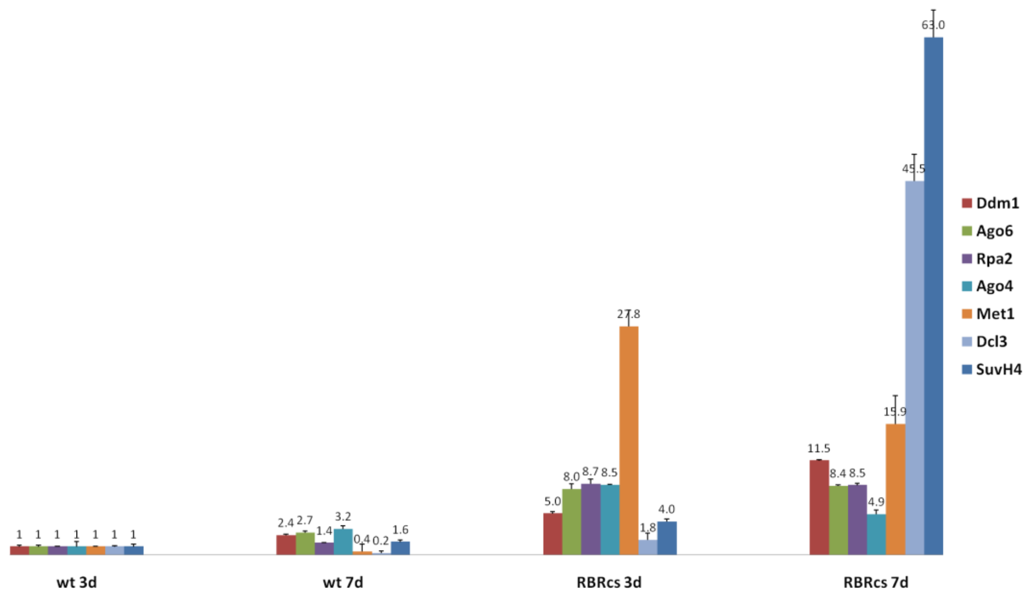


Figure1: Simplified scheme of the TGS and *de novo* DNA methylation pathway. Genes that were found with elevated expression in *RBRcs* mutants are marked with an asterisk.

gene name	locus	induction in RBRcs	up in E2Fa/Dpa?	induction in E2Fa/Dpa	E2F-site?	E2F sequence	position
Fas2	AT5G64630	16.5	yes	10.8	yes	TTTGCCGG/TTTCCCCC/TTTCGCGC	880;544;18
Rpa2	AT2G24490	12	yes	5.4	yes	ATTGGCGG/ACTCGCGG	92;375
Fas1	AT1G65470	10.4	yes	8.9	yes	TTTGGCGC/TTTCCCCC/TTTCCCGC	178;254;117
E2FC	AT1G47870	10.2	yes	9	yes	TTTCGCCC	105
Ddm1	AT5G66750	9.9	yes	12.3	yes	ATTCGCGG/TTTCCCGC	666;552
SuvH4	AT5G13960	7.8	yes	8.2	yes	TTTCCCGG	69
Cmt3	AT1G69770	7.4	yes	3.7	yes	ATTCCCGG/TTTGGCGC/TTTCGCCC/TTACGCGC	363;79;55;939
Ago6	AT2G32940	7.1	no		yes	TTTCGCGC	341
Rrd2	AT4G11130	4.9	no		no		
Drm1	AT5G15380	4.9	no		no		
E2FB	AT5G22220	3.9	yes	2.8	no		
E2FA	AT2G36010	3.4	yes	737.7	no		
Ago4	AT2G27040	3.1	no		yes	TTTGCCCG	222
Met1	AT5G49160	3	yes	8.2	yes	TTTAGCGC/ATTGCCGC	132;119
Dcl3	AT3G43920	2.9	no		no		

Table1: Shown are E2Fs and genes that are involved in TGS and display elevated expression in 3d old *RBRcs* seedlings. Induction represents fold change compared to wild type. Many of these genes contain one or several E2F-binding sites in their promoter and are also upregulated in seedlings that constitutively overexpress *E2Fa* and *DPa*. Although *E2Fa* is much stronger expressed in *E2Fa/DPa* overexpressing seedlings many of the genes that are upregulated in both mutants have higher or similar expression levels in *RBRcs*. Indicated is also the E2F binding sequence and position in base pairs upstream of the translation start.

A



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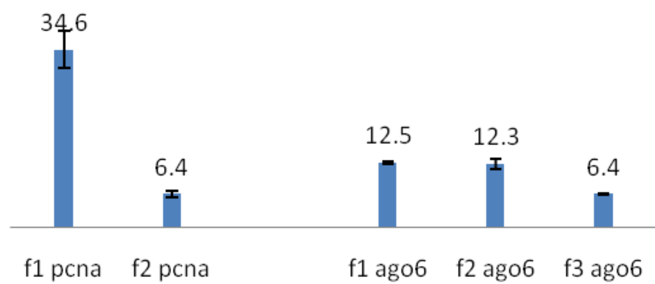


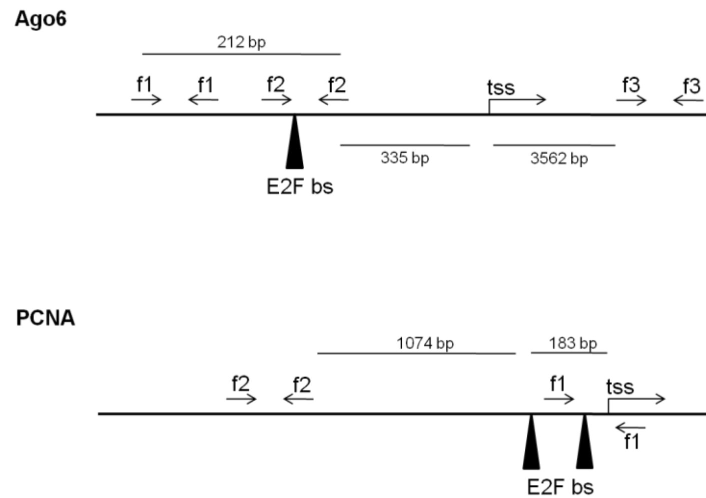


Figure2:

A: Elevated expression of genes involved in TGS and that were upregulated on microarrays with *RBRcs* mutants was confirmed via real-time-pcr. The numbers are relative expression levels compared to the wild type (3d old wild type is normalized to one). All genes were upregulated already in 3d old *RBRcs* mutant and accumulated more in 7d old *RBRcs* mutants. Error bars represent standard deviations from two technical replicas.

B: Enrichment of RBR at E2F binding sites on promoter regions of *PCNA* and *AGO6*. Chromatin was extracted from cell cultures one week after sub-culturing and used for chromatin-immuno-precipitation with α-RBR antibodies. DNA fragments were amplified and quantified in a real-time pcr experiment. Values are relative derived from the ratio of signal versus background and control antibodies (against an artificial storage protein). Errors represent standard deviations of two technical replicates.

The panel at the bottom on shows the position of the primers on the promoters of *PCNA* and *AGO6* relative to the transcription start site (tss) and putative E2F binding sites (E2F bs). The position of primers for PCNA was chosen according to Sekine et al.

The silencing pathway and de novo methylation is inaktiv in RBRcs mutants

Arabidopsis mutants defective in transcriptional gene silencing (TGS) show certain distinct characteristics (for an overview see: (Elmayan et al. 2005)). For example *DCL3*, *NRPD1a*, *NRPD2a* and *RDR2* are required for the production of siRNAs that guide DNA methylation and silencing. AGO4, CMT3, DDM1, DRM2 and MET1 modify DNA or chromatin and have an effect on the accumulation of some endogenous siRNAs. Therefore mutants do not produce the respective small RNAs and show modified DNA methylation patterns. Other mutants (*fas1*, *fas2*, *rpa2*, *mom1*, *bru1*) are defective in DNA break repair and transcriptional gene silencing. These mutants are hypersensitive to genotoxic stress and show derepression of certain transposable elements.

Because of the high expression of genes involved in transcriptional gene silencing, we expected a strong suppression of transposable elements. On the expression profile of *RBRcs* mutants we could not detect significant differential expression of any transposable element present on the ATH1 array. Next we focused on known target genes of TGS. Huettel et al (Huettel et al. 2006) identified intergenic regions as targets of RNA-directed DNA methylation. They found IG/LINE, IG1, IG2 and IG5 upregulated in Pol4 mutants and in *rdr2*, *drd1* and *met1* mutants. We used identical primer sequences to investigate the expression of this regions in *RBRcs*. Figure3 shows that the expression levels of IG/LINE, IG2 and IG5 was at least five-times higher in 7d old *RBRcs* seedlings compared to 3d or 7d old wild type seedlings. Notably the increase of expression of IG/LINE and IG1 in *RBRcs* seedlings compared to 7d old wild type seedlings was almost one order of magnitude higher. Another known TGS-target that was derepressed for example in *ddm1* (Lippman et al. 2004) is a histidine phosphotransferase (At4g04402), which is located at a transposon rich region on chromosome four. While we could hardly detect At4g04402 expression in the wild type, we saw a clear expression in 3d and 7d old *RBRcs* seedlings (fig.3). However the flowering time control gene FWA that is also controlled by DDM1 was low expressed in both wild type and *RBRcs.*

Furthermore we looked for *Supermann* expression. *ago4* has been found in a screen for mutants with derepresed Supermann expression in vegetative tissue (Zilberman et al. 2003). When we looked for Superman expression in *RBRcs* mutants, we also found a strong reactivation (Figure3).

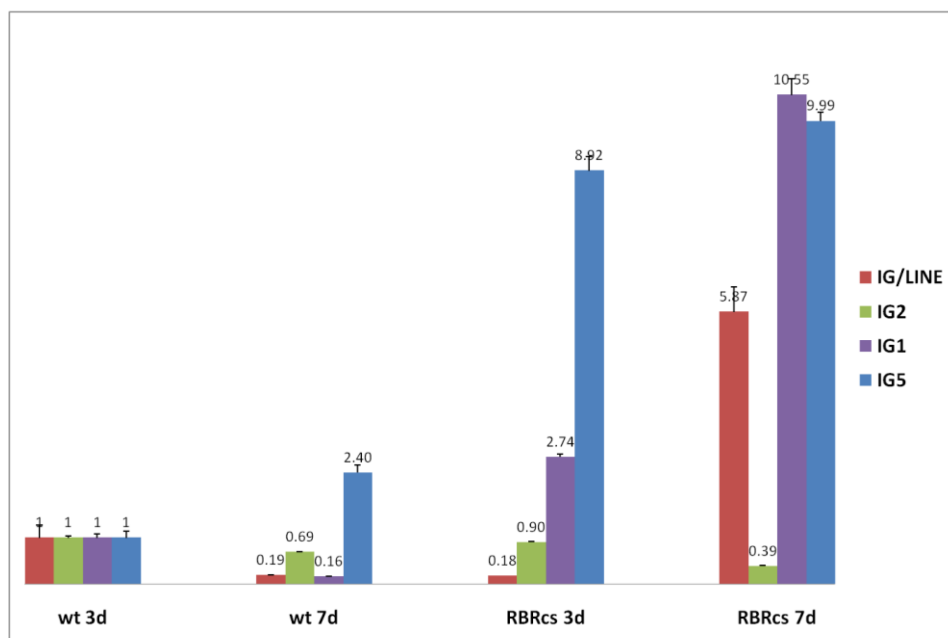
In order to show that the reactivation of these genes could be directly linked to a deactivation of the DCL3 pathway we monitored the accumulation of the 5S-repeat-derived siRNA1003. siRNA1003 is not detectable in *ago4*, *dcl3*, *drm2*, *rdr2* and pol4 mutants. At around 24 nucleotides – the size of siRNA1003 we were able to detect a signal from wild type seedlings with our probe (Figure 4). In wild type looking segregants of *RBRcs* seedlings, which have increased RBR levels compared to wild type but do not show any discernable phenotype (hence we named them *RBR-OE*), siRNA1003 accumulated much higher levels than in wild type seedlings. In *RBRcs* seedlings, we could not detect a signal at 24 nucleotides, but there was a smear of signal at a higher molecular weight that was not present in wild type or *RBR-OE* seedlings. This could suggest, that *RBRcs* seedlings were defective in correct processing of siRNA1003. To exclude the possibility that this defect of accumlation of siRNA1003 was due to a general RNA-metabolism defect in *RBRcs* seedlings we looked at another small RNA that is produced by another, microRNA processing pathway, miRNA160. We could not see a difference in the accumulation of miRNA160 in *RBRcs*, *RBRcs-OE*, or wild type seedlings. This suggested that there was a specific defect to produce siRNA1003 in *RBRcs* seedlings.

Derepression of TGS-targets and diminished siRNA accumulation in *RBRcs* mutants suggested a possible loss of cytosine methylation. To determine if in *RBRcs* seedlings cytosine methylation at the 5S gene locus was affected we performed Southern blotting using methylation sensitive restriction endonucleases and a probe directed against the 5S-rRNA encoding DNA region. HpaII and MspI cut CCGG motifs but HpaII will not cut if the second C is methylated and MspI will not cut if the first C is methylated (McClelland et al. 1994). HaeIII recognizes GGCC but cuts only if the inner C is not methylated (Onodera et al. 2005). Therefore, digestion of 5S genes with these three enzymes reports on methylation at CG, CNG and CNN. The Southern blots revealed ladders of bands at around 500bp intervals which represents the size of a 5S gene repeat (Campell et al. 1992). High levels of methylation results in a strong signal near the top, loss of methylation leads to more signal near the bottom. Figure 5 shows that there is almost no difference in CG (HpaII) and GNG (MspI) methylation of the 5S gene in *RBRcs* mutants, but there is strongly reduced CNN (HaeIII) methylation in *RBRcs* seedlings.

A common feature of the transcriptional gene silencing mutants *rpa2*, *fas1*, *fas2, mom1* and *bru1* is their sensitivity to genotoxic stress (Takeda et al. 2004) suggesting a role of these genes in both TGS and DNA repair pathways (Elmayan et al. 2005). This can be tested by growing the mutant seedlings on medium supplemented with methyl methane sulfonate (MMS), a chemical that alkylates DNA and is considered to mimic double-strand-DNA break damage (Takeda et al. 2004). Also *RBRcs* seedlings were hypersensitive to MMS despite the higher expression of many DNA-repair genes (Figure4). Also, we used gene expression information of *fas1* and *fas2* mutants (Schonrock et al. 2006) and compared it with differentially expressed genes in *RBRcs* seedlings. Figure4 shows that despite a strong overexpression of *FAS1* and *FAS2* in the *RBRcs* mutant background we found a significant positive correlation of deregulated genes in *RBRcs* and *fas1* and *fas2* mutants, which further suggests that FAS1 and FAS2 are not active in *RBRcs* seedlings.

Taken together these experiments suggest that *RBRcs* seedlings are defective in DNA repair pathways and in the production of siRNA, which results in a loss of CNN methylation and a reactivation of TGS-target genes.

A



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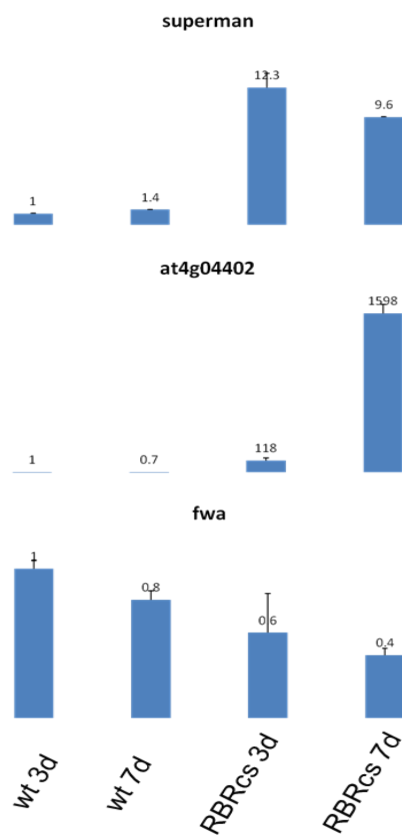

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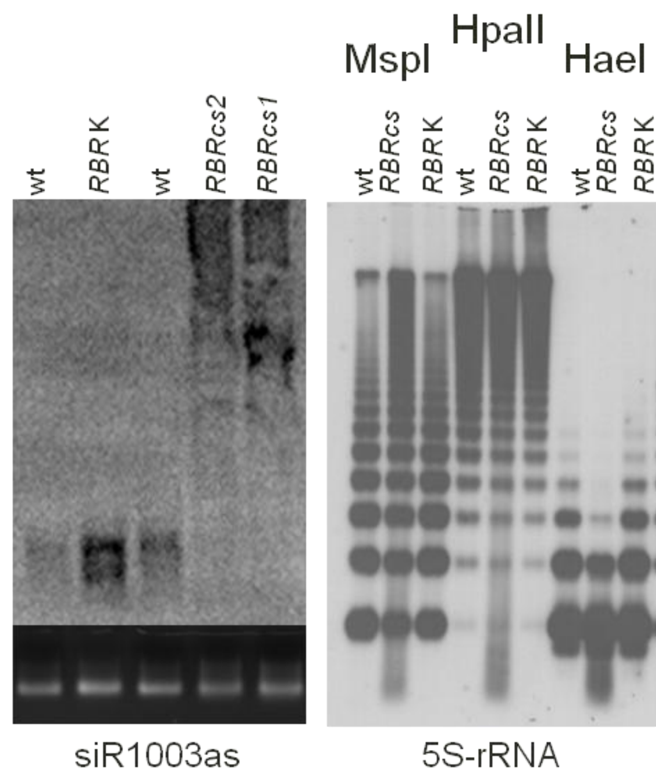

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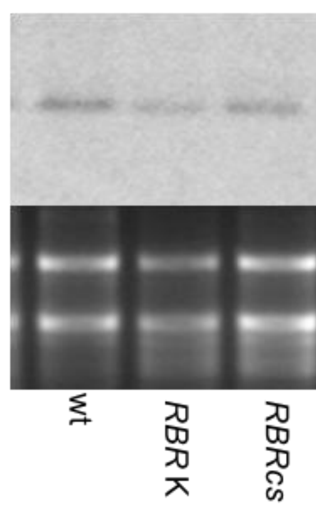


Figure3:

A: Derepression of de novo DNA methylation targets. The intergenic regions IG/LINE, IG1, IG2 and IG5 have been identified by Huettel et al.. IG1, IG5 and IG/LINE show a strong derepression in *RBRcs* mutants.

B: Derepression of other known target genes of TGS: Superman is usually only expressed in male reproductive organs but gets activated in the kryptonite mutant (SuvH4). At4g04402 is a gene close to a transposon on a region at chromosome four close to a heterochromatic knob which is usually repressed. FWA another target was very low in wild type and *RBRcs* seedlings. The numbers indicate relative expression levels - 3d old wild type was normalized to one.

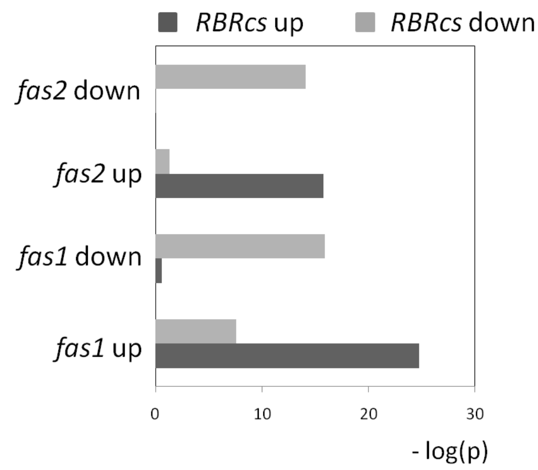
C: left: siRNA1003 is produced by the *DCL3* pathway. It was not present in *RBRcs* seedlings but in wild type control and *RBR-OE* seedlings (elevated compared to wild type). A smear in *RBRcs* seedlings could indicate defective processing of siRNA1003.

Right: A southern blot probed for 5srRNA-repeats. HaeI reports on CNG methylation and indicates that *RBRcs* mutant seedlings are less CNG methylated on 5SrRNA repeats.

D: Levels of miRNA160 were not reduced in *RBRcs* mutants. miRNA160 is not produced by the *DCL3* pathway.

RBRcs1 +2 are different *RBRcs* lines. *RBR-K* are sibling-seedlings of *RBRcs* mutants with wild type appearance.

A



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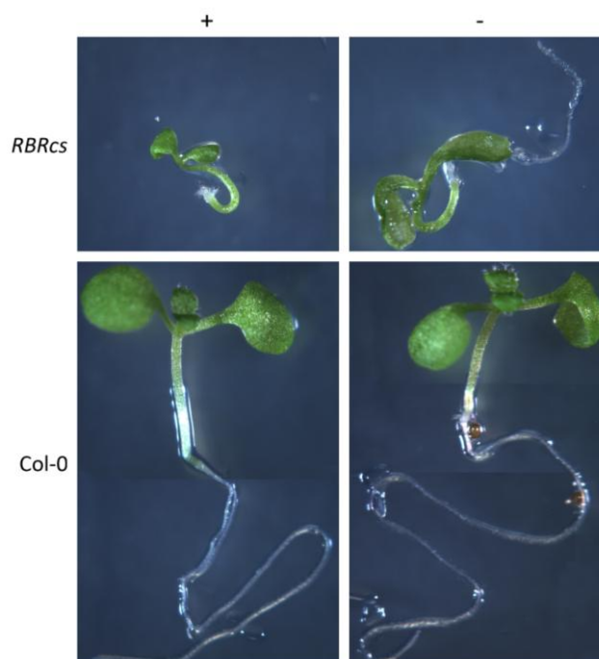


Figure4: *RBRcs* are defective in DNA repair.

A: Despite strongly elevated expression levels of *FAS1* and *FAS2*, gene expression in *RBRcs* seedlings correlates strongly with *fas1* and *fas2* mutants, suggesting a decreased activity of FAS1 and FAS2. Shown are the p-values that the overlapping number of genes were found by coincidence.

B: *RBRcs* mutant seedlings are hypersensitive to genotoxic stress. After one week on growth medium with or without 50ppm MMS (left column) Col-0 seedlings show no differenc. *RBRcs* seedlings are strongly inhibited in growth by the presence of MMS.

RBRcs seedlings contain less AGO4, AGO6 and DCL3 protein

Because of this intriguing discrepancy of expression level and activity of many genes involved in TGS we reasoned that transcripts of these genes are controlled on a posttranscriptional level. Since we had no antibodies against the proteins in questions we decided to use a proteomics approach to estimate their relative abundance. The Apex (Lu et al. 2007) factor has been successfully used to determine abundance values for proteins that have been detected by Mass-spectrometry (Baerenfaller et al. 2008). We chose to use 3d old seedlings for proteomics-profiling since we could directly compare the protein abundances with our previously performed microarray experiment. From the resulting data we were able to detect a total of 4120 proteins (3082 proteins from wild type and 3364 proteins from *RBRcs* seedlings; resulting in an overlap of 75% for wild type and 69% for *RBRcs*) via at least one significant peptide (supplementary information). From those approximately one tenth was up or down regulated on the gene chip experiment. With the APEX factor we could determine which of these proteins were up or downregulated in *RBRcs* compared to wild type. Next we compared the results from the proteome profiling with the expression profiling. The relative abundance of most proteins correlated with their expression in *RBRcs* mutants. 87% of proteins with a higher apex factor in *RBRcs* than in wild type also had significantly higher mRNA levels. And 85% of proteins with a lower apex factor in *RBRcs* than in wild type had significantly lower mRNA levels (Table2). The only proteins of the *de novo* methylation pathway that we could detect with our proteomics approach were AGO4, AGO6 and DCL3. Notably these 3 proteins were amongst 39 proteins with a lower apex factor in *RBRcs* seedlings than wild type but with significantly higher mRNA levels (Table3). This implies that despite increased mRNA levels of *AGO4*, *AGO6* and *DCL3* the protein levels of these genes were actually lower in *RBRcs* mutants compared to wild type seedlings. This strongly suggests disturbed posttranscriptional control mechanisms of *AGO4*, *AGO6* and *DCL3* in *RBRcs* seedlings.

This was reminiscent of *RBR*. In *RBRcs* mutants RBR protein was strongly reduced but mRNA levels were strongly increased compared to wild type. This is illustrated in Figure5 and was confirmed with western-blot analysis, quantitative PCR and northern-blot analysis. Furthermore we could also downregulate RBR protein when we supertransformed *RBR* promoter-GUS transcriptional fusion marker lines with a hairpin-construct directed against RBR. These constitutive RNA interference lines displayed the same phenotypic abnormalities as *RBRcs* mutants, contained less RBR protein and showed increased *RBR* expression. These mutants showed strongly increased GUS staining compared to non-transformed marker-lines (Figure5).

RNA interference takes place in the cytoplasm (Dudley and Goldstein 2003) and in plants, transgene silencing results mostly in degredation of the targeted mRNA.

Since our hairpin construct resulted likely in RNA interference we reasoned that *RBR* mRNA might be protected from degradation because it would accumulate in the nucleus.

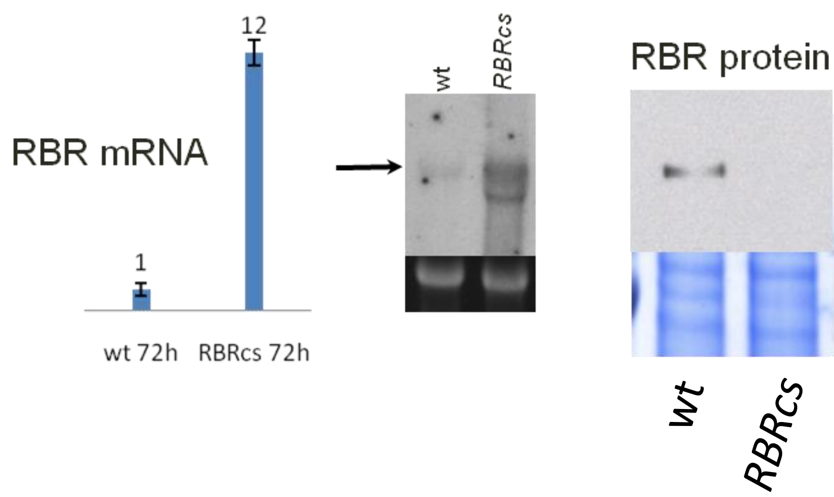
	prot up (295)	prot down (369)
mRNA up (1586)	256 (87%)	39 (13%)
mRNA down (1789)	54 (14%)	315 (85%)

Table2: Shown is the overlap of mRNA that has been found on *RBRcs* microarrays and protein from proteomics-profiling. 87% of genes with increased expression showed a positive correlation of mRNA and protein abundance as did 85% of genes with a decreased expression.

locus	wt unique	wt tot num	wt apex	RBRcs apex	RBRcs unique	RBRcstot num	description	expression diff.
AT1G07790	8	19	6.19	0.00	0	0	HISTONE H2B	2.10
AT3G21720	35	127	12.99	9.21	27	79	isocitrate lyase, putative	4.65
AT3G20670	6	8	3.58	1.53	3	3	DNA binding	3.02
AT3G06860	41	84	5.79	4.55	30	58	MFP2; enoyl-CoA hydratase	3.19
AT5G11520	21	36	4.03	2.81	11	22	ASPARTATE AMINOTRANSFERASE 3	1.67
AT4G30320	2	2	1.19	0.00	0	0	Tpx-1-related family protein	1.44
AT5G42890	11	18	5.86	4.82	12	13	sterol carrier protein 2	1.92
AT2G16060	2	3	0.72	0.00	0	0	ARABIDOPSIS HEMOGLOBIN 1	2.56
ATMG00640	3	5	1.49	1.02	3	3	ORF25	1.23
AT5G02530	7	7	1.32	0.86	4	4	RNA and export factor-binding protein, putative	1.08
AT3G05190	5	5	0.44	0.00	0	0	aminotransferase class IV family protein	2.39
AT3G16320	3	5	0.42	0.00	0	0	binding	1.83
AT2G05710	38	62	3.42	3.01	28	48	aconitate hydratase, cytoplasmic, putative	2.21
AT3G60660	2	2	0.38	0.00	0	0	similar to unknown protein	1.80
AT4G39510	3	3	0.32	0.00	0	0	CYP96A12	1.42
AT1G55915	2	2	0.29	0.00	0	0	zinc ion binding	1.44
AT1G32900	3	3	0.26	0.00	0	0	starch synthase, putative	2.52
AT4G31840	3	3	1.07	0.82	2	2	plastocyanin-like domain-containing protein	1.58
AT4G32920	3	5	0.26	0.00	0	0	glycine-rich protein	1.33
AT2G22780	11	13	2.02	1.77	8	10	PEROXISOMAL NAD-MALATE DEHYDROGENASE 1	1.41
AT3G10690	11	14	0.85	0.62	7	9	DNA gyrase subunit A family protein	1.16
→ AT2G32940	4	4	0.20	0.00	0	0	ARGONAUTE 6	2.83
AT5G41580	2	2	0.14	0.00	0	0	zinc ion binding	1.51
→ AT3G43920	4	4	0.14	0.00	0	0	DICER-LIKE 3	1.57
→ AT2G27040	6	14	0.74	0.60	10	10	ARGONAUTE 4	1.64
AT5G23150	3	3	0.11	0.00	0	0	HUA2 (ENHANCER OF AG-4 2)	1.24
AT3G03110	3	4	0.23	0.13	2	2	XPO1B (exportin 1B)	1.07
AT4G22970	2	3	0.08	0.00	0	0	AESP; ARABIDOPSIS HOMOLOG OF SEPARASE	4.10
AT4G15890	4	11	0.47	0.39	3	8	binding	1.68
AT2G37770	3	3	0.34	0.26	2	2	aldo\keto reductase family protein	3.40
AT5G49110	2	2	0.07	0.00	0	0	similar to hypothetical protein	3.27
AT1G50240	5	6	0.23	0.17	3	4	armadillo\beta-catenin repeat family protein	3.40
AT3G59100	2	2	0.05	0.00	0	0	GLUCAN SYNTHASE-LIKE 11	2.54
AT4G02280	4	4	0.28	0.24	3	3	UDP-glycosyltransferase	4.06
AT3G23800	6	6	0.69	0.66	5	5	SBP3 selenium-binding family protein	1.48
AT1G08260	5	5	0.10	0.07	3	3	EMB2284, EMB529	2.32
AT4G38440	4	4	0.15	0.13	3	3	similar to hypothetical protein	1.27
AT4G29900	4	6	0.36	0.34	3	5	calcium-transporting ATPase	1.28
AT2G34680	6	6	0.18	0.17	5	5	Auxin-Induced in Root cultures 9	1.32

Table3: Genes with elevated expression but lower protein abundance in *RBRcs* mutant seedlings. The only proteins of the *DCL3* and DNA *de novo* methylation pathway that we found in this proteomics approach were DCL3, AGO4 and AGO6. All three were found is this category of genes.

A



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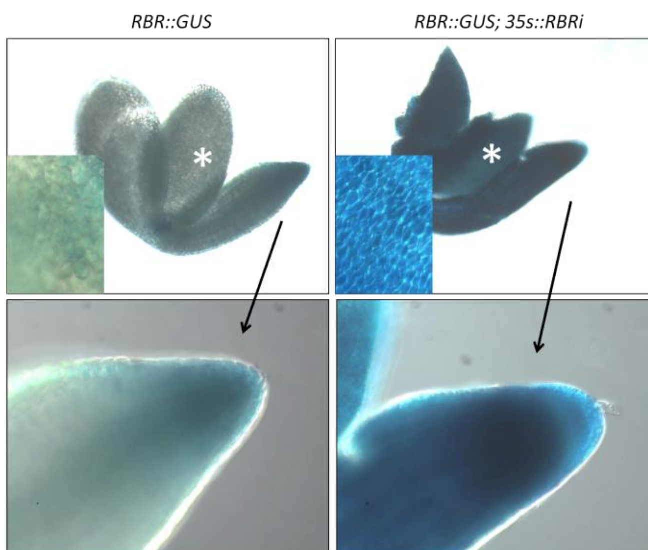


Figure5:

RBRcs and *35S::RBRi* contain less RBR but accumulate more mRNA than wild type seedlings.

A: Shown is mRNA quantification of *RBR* with real-time-PCR and northern blot analysis. A western blot shows reduced RBR protein levels in *RBRcs* mutants.

B: 1d old *35S::RBRi* seedlings in *RBR::GUS* background show strong GUS staining compared to wild-type *RBR::GUS* seedlings.

mRNA of AGO6 and RBR accumulated in the nucleus of *RBRcs* seedlings

In order to test this hypothesis, we isolated nuclei from 7d old *RBRcs*, *RBR-OE*, wild type and 3d old wild type seedlings and extracted RNA. In parallel we extracted RNA from whole seedlings. We could measure a slight accumulation of *AGO6* and *RBR* mRNA in RNA isolated from wt and *RBR-OE* nuclei but a much stronger accumulation in nuclei of *RBRcs* seedlings (Figure6). To exclude that this accumulation of RNA was just an effect of an overall higher transcription rate we included *PCNA*, which was also highly overexpressed in *RBRcs* seedlings but displayed a positive correlation of transcript and protein abundance. We could not detect strong enrichment of PCNA mRNA in RNA of nuclei from *RBRcs* mutants.

These results could suggest that transcript of *RBR* and *AGO6* accumulated in *RBRcs* seedlings because it accumulated in nuclei and hence cannot be translated.

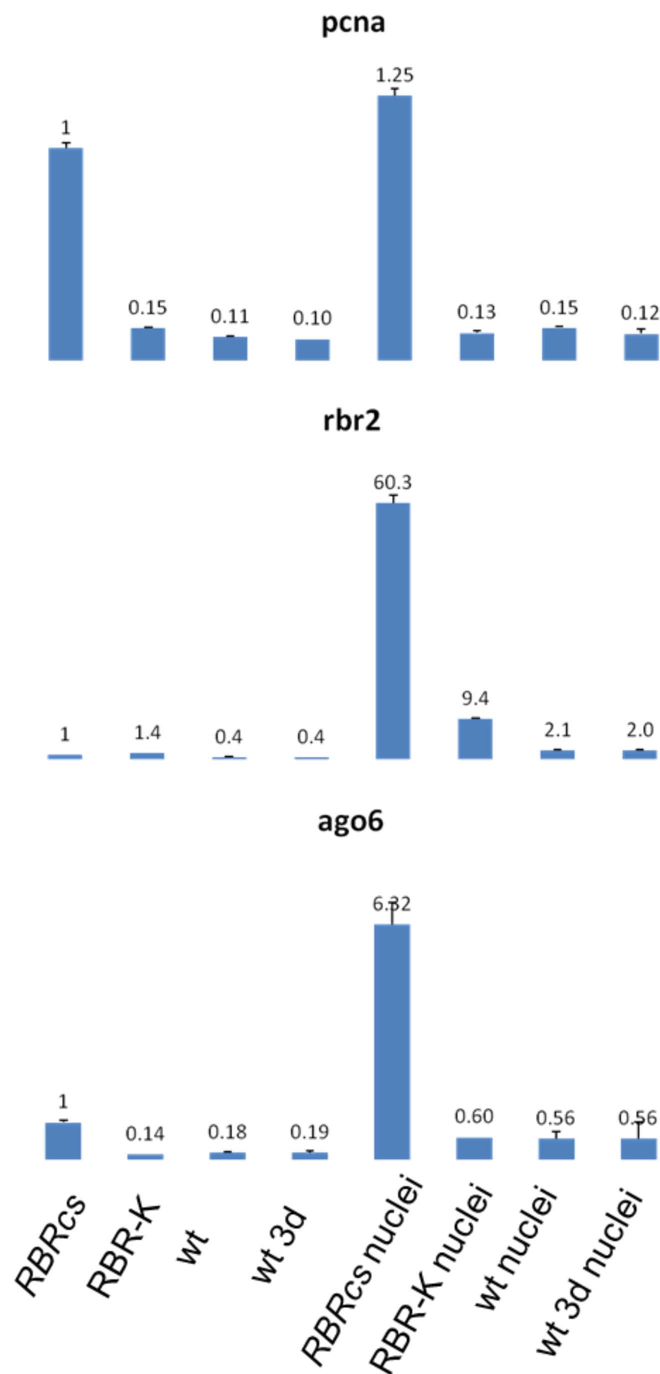


Figure6: *RBR* mRNA accumulated in nuclei of *RBRcs* mutant seedlings. RNA was extracted either from whole seedlings or from isolated nuclei. *RBR* and *AGO6* showed strong accumulation of transcript in nuclei of *RBRcs* seedlings. *PCNA* which was similarly high expressed as *RBR* and *AGO6* did not accumulate in nuclei. *RBR-K* are sibling-seedlings of *RBRcs* mutants with wild type appearance.

Discussion

The experiments reported here begin to identify the connections of the RBR pathway with RNA interference and transcriptional gene silencing in *Arabidopsis*. In mutants with reduced levels of RBR we found a strongly increased expression of genes important for DNA-synthesis and nucleic acid metabolism. Many of these genes contain E2F sites in their promoters what suggests that E2F transcription factors can bind to these regions, especially, since many S-phase genes are regulated by E2F. Hence for the DNA-maintenance methylation pathway this result may be not so surprising because newly synthesized DNA needs to adopt the same methylation pattern as the parent strand. However this does not include de novo methylation. We found most of the genes required in the *DCL3* transgene silencing/de novo methylation pathway expressed at higher levels in *RBRcs* mutants than in wild type. We found an enrichment of the *AGO6* promoter region when we precipitated chromatin from cell suspension culture with α-RBR antibodies. If the other genes are also directly regulated by RBR and whether binding of E2F is necessary to activate transcription of these genes remains to be determined. In a recent study which used whole-genome tiling arrays many E2F sites on promoters have been newly defined (Naouar et al. 2009). Interestingly, among the list of genes with predicted E2F sites are many transposable elements, which could indicate a direct regulation of the repression of transposable elements via the RBR/E2F pathway. From the upregulated expression of genes in the silencing pathway we expected – similar to the situation in *C.elegans* - an increase in activity of the Dcl3/DNA de Novo methylation pathway. First we looked at the transcription levels of targets of the polymerase IV subunits and found surprisingly an upregulation of these transcripts which indicates a decrease of activity of the silencing pathway. The upregulated transcripts identified by Huettel et al. represent plant genes and intergenic regions located in euchromatin (Huettel et al. 2006). Notably, the three intergenic targets are adjacent to short RNA-encoding sequences. For IG2 and IG5 this sequence is a LTR retrotransposons. Interestingly for IG2 – where we do not see a difference compared to wild type the truncated Athila solo LTR is in opposite orientation to the IG2 transcript whereas the strong derepressed IG5 transcription initiates within the Copia LTR. The lack of transpososns in the 5 prime flanking regions of IG1 might reflect incomplete annotation of the *Arabidopsis* genome or a 5 prime flanking region might be bidirectionally transcribed, producing overlapping sense and antisense transcripts that could form double-stranded RNA as a trigger for silencing (Huettel et al. 2006). Also the promoter of the flowering time gene *FWA* contains a transposon-derived repeat that is the target of short RNA-mediated silencing via DDM1 (Lippman et al. 2004). Although we found no significant change of *FWA* expression in RBRcs seedlings we found a gene similar to a histidine phosphotransferase strongly derepressed. In wild type this gene is silent, heavily methylated and associated with H3mK9 (Lippman et al. 2004). Within the

gene a *Vandal* transposable element is inserted but in DDM1 mutants both the transposon and the gene are activated. The location of this gene was within a heterochromatic knob, a repetitive region associated with H3mK9 methylation pattern (Lippman et al. 2004). These genes seem not to be changed in pol4 mutants which suggest that *Arabidopsis* has probably more than one pathways that use short RNAs to induce transcriptional gene silencing. Either RBR influences both pathways in a particular way or acts on another silencing pathway. Another marker that we used as readout for the activity of transgene silencing/de novo methylation was transcript levels of superman. Superman was reactivated in ago4 mutant background where the locus also showed decreased CpNpG and asymmetric DNA methylation as well as histone H3 lysine-9 methylation (Zilberman et al. 2003).

Thus we see a derepression of various genes in *RBRcs* mutants that we would expect to find in knockout alleles of genes of the *DCL3*/*de novo* methylation pathway. In *Arabidopsis* are at least two systems to generate distinct classes of endogenous small RNAs (Xie et al. 2004). The miRNA-generating system requires *DCL1*, but not RDR proteins (Park et al. 2002; Reinhart et al. 2002). The second system requires *DCL3* and *RDR2* to generate endogenous siRNAs. We chose to test two exemplary representatives for each system. miRNA160 targets auxin response factors *ARF10, ARF16* and *ARF17* and plays an important role in many developmental processes (Wang et al. 2005). The endogenous small RNA 1003 originates from 5S rDNA repeats. The 5S rRNA genes occur in tandem arrays with a typical repeat unit of transcribed and flanking sequences. Probes against siRNAs usually detect populations of 21-24 nucleotids species. The abundance of miRNA160 was not changed in the RBRcs mutant, compared to wild type, which indicates that the miRNA producing pathway is not disturbed in RBRcs. This was in contrast to siRNA1003. In *RBRcs* seedlings we were not able to detect a small RNA with our probe against siRNA1003 but we detected a strong signal at a higher molecular weight. The repeat units of siRNA1003 are approximately 500 nucleotides and the transcribed sequence 120 nucleotides long which mean that a disturbed processing could potentially result in a smear smaller than 120 base pairs. However such a phenomenon has not been reported in siRNA-pathway mutants such as *dcl3, ago4, ago6* or *rdr2*. Either this does not occur in mutants without activity or the signal in *RBRcs* mutants is due to unspecific probe-annealing. Interestingly, in *RBR-OE* mutants, which have an increased level of RBR protein we could detect an accumulation of siRNA1003 compared to wild type. However we did not find a significant change in transcript levels for any of the siRNA pathway genes in *RBRcs*-control seedlings. Because of the number of 5S rDNA repeats analysis of cytosine methylation was done using methylation sensitive restriction enzyme digestion. In the *dcl3*-mutant cytosine methylation is decreased only at asymmetric sites while CG and CNG methylation is similar to wild type (Xie et al. 2004). In mutants lacking chromomethylase3, AGO4 or RDR2 the methylation at CG sites is only slightly reduced, but it is stronger reduced for CNG and CNN methylation (Zilberman et al. 2003; Xie et al. 2004). In *RBRcs* mutants we found almost

no change for CG and CNG methylation (HpaII and MspI respectively) but we found a clear reduction in CNN methylation (HaeIII). Therefore reducing levels of RBR in *RBRcs* mutants affected cytosine methylation only in a CNN sequence context which is similar to mutants of the siRNA/de novo methylation pathway. These facts are most parsimonious with the hypothesis that RBR is a positive activator of the production of siRNA and establishment of CNN cytosine methylation at repeated sequences.

This is why we suspected a posttranscriptional regulation of these mRNAs. Therefore we decided to quantify the proteins involved in siRNA production. Absolute protein expression measurements (APEX) is a robust and rapid method to quantify absolute protein abundance, without requiring construction of fusion protein libraries, labeling or internal standards (Lu et al. 2007). Differential protein levels in RBRcs mutants correlated well with differential mRNA levels. The only proteins of the siRNA production pathway for which we were able to detect significant peptides were AGO4, AGO6 and DCL3. Furthermore the Apex factor of these proteins suggested a lower abundance in RBRcs mutants than in wild type. This is in contrast to strongly increased mRNA levels of these proteins, suggesting a posttranscriptional regulation of translation of these mRNAs. This was very similar to what we actually found for RBR. In RBRcs mutants we found a strong accumulation of full-length *RBR* mRNA but a significant reduction of RBR protein. Therefore we assumed that the hypotethysed posttranslational modification would be a direct result of RBR downregulation. The human retinoblastoma-binding proteins 48 and 46 (RbAp48 and RbAp46 respectively) have been found associated with the nuclear-pore-complex in human cells (Cronshaw et al. 2002). Both are orthologs to the *Arabidopsis* protein MSI1 which has been shown to interact with RBR in vitro and in vivo (Jullien et al. 2008). Also importin alpha and importin beta have been found at the nuclear pore complex, for which we could show a direct binding to RBR in vivo. Interestingly, in human cells, the beta-like import receptor Imp8 has been found to bind to Ago1-Ago4, to colocalize with Argonaute proteins in P Bodies and to be involved in loading Ago complexes onto mRNA targets. Therefore we concluded that RBR might influence the regulation of probably its own expression and of genes for the siRNA pathway by influencing the activity of nuclear import-export of specific mRNAs. Comparing cytoplasmic and nuclear mRNA accumulation of *RBR* and *Ago6* showed that there was a strong accumulation of mRNA of *RBR* and *AGO6* in the nucleus but not for *PCNA*. This could suggest that mRNAs of *RBR* and *AGO6* are indeed less translated because they accumulate in the nucleus and cannot efficiently be transported into the cytoplasm. Although the mechanisms are not at all clear at the moment we found a very interesting negative correlation of mRNA and protein abundance for RBR and proteins of the siRNA/de Novo methylation pathway.

Material and methods

Plant material and growth conditions

Construction of plant lines with reduced levels of RBR was described before. For all experiments seeds were sterilized according to standard methods, and stratified for 4 nights at 4° in the dark on growth medium, which consisted of MS medium supplemented with either 1% sucrose or equivalent-molar amount of mannitol as control and for MMS 50ppm were added after autoclaving. Plates were put into Conviron growth chambers (mixed fluorescent an incandescent light 230µmol/m2/s at 22°) under long day condition (16h light).

Protein gel blot analysis

Protein extracts were prepared from *Arabidopsis* by grinding shock-frozen tissue. Subsequently extraction buffer was added (7M urea, 2M thiourea, 10% v/v isopropanol, 5% v/v glycerol, 2% v/v pharmalyte, 50mM DTT, 1xComplete protease inhibitor cocktail (Roche)). Homogenates were centrifuged 2x20min at RT. Protein concentration was equilibrated (using a simple bradford method with the Roth-Nanoquant-solution according to manufacturers protocol) until all samples contained the same concentration. Laemmli-buffer was added and 100µg of protein was added to each lane of an 8% SDS PAGE. For each Western-blot in parallel as loading control another gel was prepared which was subsequently coomassie stained according to standard procedures. Blotting was performed semi-dry onto nitrocellulose in 20% v/v MeOH, 0.29% w/v glycine, 0.58% w/v Tris-base, 0.04% w/v SDS at 0.2V/cm2 for 2h. The membrane was incubated over night at 4° in TBST (150mM NaCl, 50mM Tris pH 7.5, 0.1% Tween20) with 5% w/v dry milk powder. Blots were subsequently incubated for 3h with a 1:400 dilution of α-RBR antibody in TBST plus milk. After 3x10min washing in TBST secondary α-rabbit antibody 1:5000 in TBST plus milk was added and the blot was incubated for another 2h. After 4x final washing chemiluminescent detection ws performed with the ECL-enhancer kit from Bio-rad according to manufacturer`s instructions.

RNA isolation and Q-PCR

RNA was extracted using Trizol (Invitrogene) according to manufacturers instructions. For Q-PCR, RNA was treated with DNase I. 2µg of RNA was reverse-transcirbed using oligo(dT) primers and superscript (Invitrogene). Aliquots of the generated cDNA were used as template for PCR with gene specific primers (table material and methods). Q-PCR was performed in an ABI Prism 7700 Sequence Detection system (Applied

biosystems AB), using FAST SYBR Green Master Mix reagent (AB) according to the manufacturer's instructions. All amplification plots were analyzed with a fluorescent signal threshold of at least 0.1 to obtain Cycle Treshold values. Experiments were performed in duplicate with error bars representing the range. Gene expression levels were normalized to pp2a as control gene, which was the most stable gene in seedlings according to the Biomarker discovery tool from Genevestigator (Zimmermann et al. 2004). For Northern-blot analysis a full-length RBR probe was generated and labeled with the DIG-system from Roche (http://www.roche-applied-science.com/PROD_INF/BIOCHEMI/no3_03/PDF/p13_15.pdf). Membrane transfer and detection was performed according to standard and manufacturers protocols. Southern blot was performed as described in (Kanno et al. 2005) but with DIG labeled probes. Northern blot analysis for small RNA was performed according to (Akbergenov et al. 2006).

Chip

CHIP was performed as previously described (Bowler et al. 2004). Affinity-purified α-RBR antibodies were used for precipitation. Immunoprecipitated DNA was analyzed by quantitative PCR as described above.

Microarray hybridization and evaluation

The microarray experiment was described before. Significance of overlaps of gene-sets were calculated with an hypergeometric distribution and R.

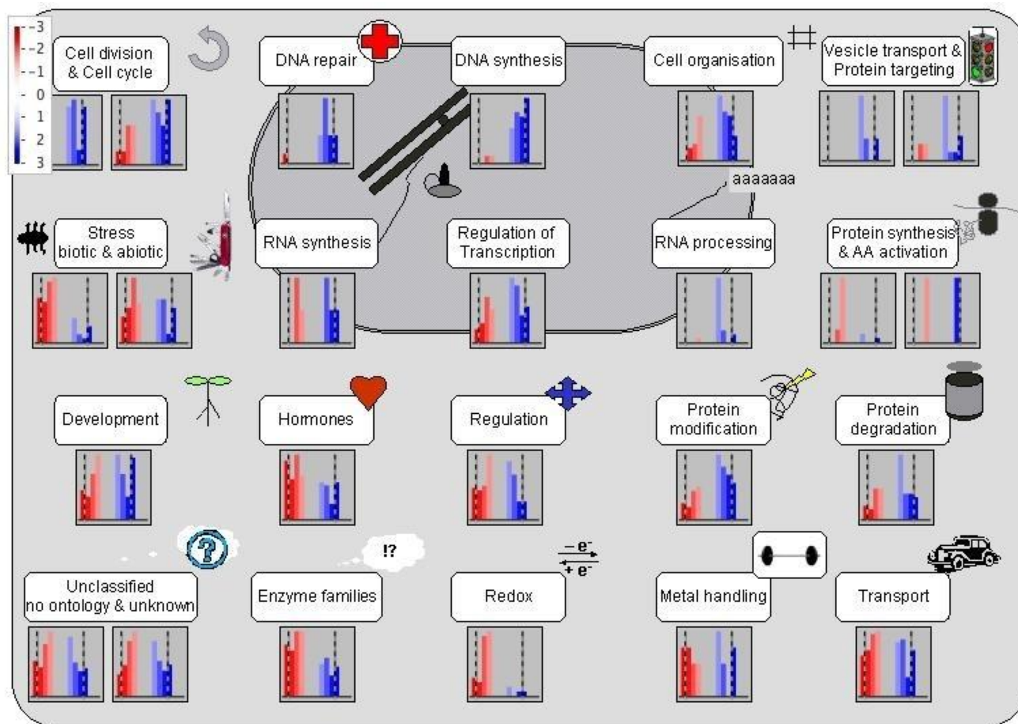
Protein detection with mass-spectrometry

Equal amounts of 3d old shock-frozen seedlings were grinded and dissolved in extraction buffer (50mM Tris-HCL pH 6.8, 50mM NaCl, 4% SDS, 5% v/v Glycerol). Aliquots were diluted and protein concentration was determined using a BCA Protein Assay Kit (Thermo scientific). 50µgProteins/lane were subjected to SDS PAGE on 12% gels. After electrophoretic separation of the proteins, the gels were cut into 10 pieces for each fraction. Each gel slice was diced into small pieces. In gel digestion was performed according to (Shevchenko and Shevchenko 2001).

Mass spectrometry measurements were performed on an LTQ FT-ICR (Thermo Finnigan), coupled with a Probot (LC-Packings/Dionex) autosampler system and the UltiMate HPLC-system (LC-Packings/Dionex). Peptide mixtures were loaded onto laboratory made capillary columns (75 µm inner diameter, 8 cm length, packed with Magic C18 AQ beads, 3 µm, 100 Å (Microm)). Peptides were eluted from the column by an increased acetonitrile concentration in the mobile phase from 5% acetonitrile, 0.2%

formic acid to 40% acetonitrile, 0.2% formic acid over 75 minutes, followed by a 10 minute wash step at 5% acetonitrile, 0.2% formic acid. Peptide ions were detected in a survey scan from 300 to 1'600 amu followed by 3 data-dependent MS/MS scans (isolation width 2 amu, relative collision energy 35%, dynamic exclusion enabled, repeat count 1, followed by peak exclusion for 2 minutes).
Interpretation of MS/MS spectra and data filtering MS/MS spectra were searched with TurboSequest and PeptideProphet by using the Trans-Proteomic Pipeline (TPP v2.9) against the *Arabidopsis thaliana* TAIR8 protein database (download on December 14th 2007) supplemented with contaminants. The search parameters were: requirement for tryptic ends, one missed cleavage allowed, mass tolerance = -/+ 3 Da, variable modification of methionine (M, PSI-MOD name: oxidation, ModAccession: MOD:00412, mono $\Delta = 15.9949$) and static modification of cysteine (C, PSI-MOD name: iodoacetamide derivative, ModAccession: MOD:00397, mono $\Delta = 57.021464$).
For PeptideProphet, the cutoff was set to a minimum probability of 0.9. APEX factors were determined according to (Lu et al. 2007).

Histological and Cytological analysis
GUS staining of transgenic plants was performed following a modified protocol from (Sieburth and Meyerowitz 1997). Plant tissues were treated with cold 90% v/v acetone, and incubated for 2h at 37°C in X-Gluc staining solution (50mM NaPo4, 5mM K3Fe(CN)6, 5mM K4Fe(CN)6, 5mM X-Gluc). Chlorophyll was removed using an ethanol series from 30% v/v to 100% v/v (each step 1h). Tissues were cleard with 50% to 100% v/v Roth-Istol (Roth) mounted on oil and observed with a Zeiss Axioplan microscope.



Supplementary figure1: We used the public available tool mapman (Thimm et al. 2004) to visualize gene expression differences of *RBRcs* and wild type seedlings. Genes are sorted in bins and these bins are assigned to cellular functions. Genes for DNA syntheses, DNA repair, cell division and RNA processing were significantly upregulated in *RBRcs* mutants. Each column represents several genes, for example the four columns of upregulated genes for DNA synthesis represent 85 genes out of a total of 364 genes that were assigned to the cellular function DNA synthesis. This means a highly significant (-log(p)>20) enrichment of this class of genes.

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6. Concluding remarks

In recent years, our knowledge of the mechanisms regulating cell cycle and endoreduplication has been considerable improved. Nevertheless, relatively little is known of how the cell cycle machinery communicates with intrinsic, developmental and positional signals and external, environmental, hormonal and nutritional cues. Here we show that RBR is an important player in these processes connecting cell fate determination with external sucrose signaling. Future research will elucidate the exact mechanisms how RBR is mediating this connection and whether this is a special role of RBR during germination or a more general role in plant cell differentiation.

Furthermore, with a multifunctional protein like RBR it is very important to exactly define how it interacts with other proteins and to decipher how its activity is regulated on a transcriptional and post-transcriptional level. For plant RBRs this research topic is still in its infancy. However the field is moving with rapid pace and the understanding of the function of RBR will with certainty also improve our understanding of fundamental developmental processes.

7. Curriculum Vitae

Personal data born on the 14th of July 1977 in Innisfail (Australia)

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06/2004 - **Graduate student** within the Molecular Life Science (MLS) PhD-Program of the Swiss Federal Institute of Technology (ETH) Zurich, Switzerland.

Education

04/2004 Completion of **diploma** (MS-homolog :o) in Biology;
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05/2003 - 04/2004 **Diploma theses** in Microbiology in the laboratory of
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"cloning, expression and purification of the trehalose, maltose and sucrose ABC transporter from Thermus thermophilus"

2002/2003 Coursework and examinations towards my diploma degree -
Undergraduate Tutor: running tutorials in cell biology and mathematics. University of Konstanz

2001 **Studies abroad**: Postgraduate courses in Botany -
University of the Western Cape, South Africa
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The high-affinity maltose/trehalose abc transporter in the extremely thermophilic bacterium Thermus thermophilus HB27 also recognizes sucrose and palatinose.

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