Review

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Roles of plant retinoblastoma protein: cell cycle and beyond

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Abstract

The human retinoblastoma (RB1) protein is a tumor suppressor that negatively regulates cell cycle progression through its interaction with members of the E2F/DP family of transcription factors. However, RB-related (RBR) proteins are an early acquisition during eukaryote evolution present in plant lineages, including unicellular algae, ancient plants (ferns, lycophytes, liverworts, mosses), gymnosperms, and angiosperms. The main RBR protein domains and interactions with E2Fs are conserved in all eukaryotes and not only regulate the G1/S transition but also the G2/M transition, as part of DREAM complexes. RBR proteins are also important for asymmetric cell division, stem cell maintenance, and the DNA damage response (DDR). RBR proteins play crucial roles at every developmental phase transition, in association with chromatin factors, as well as during the reproductive phase during female and male gametes production and embryo development. Here, we review the processes where plant RBR proteins play a role and discuss possible avenues of research to obtain a full picture of the multifunctional roles of RBR for plant life.

Keywords Arabidopsis; CDK/cyclin; cell differentiation; chromatin; endoreplication

Subject Categories Cell Cycle; Plant Biology

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Introduction

Formation of organs, either during embryogenesis as in animals or post-embryonically as in most plants, relies on a continuous supply of new cells. Failure to properly coordinate cell division, cell cycle exit into cell differentiation, and cell fate acquisition frequently results in abnormal growth, developmental aberrations, or cell transformation and tumorigenesis. Strict control of cell cycle progression is necessary to achieve the goal of producing two daughter cells. A plethora of studies has demonstrated that progression through G1 phase and into S-phase, as well as the G2-to-M and metaphase-to-anaphase transitions, represents key checkpoints during the cell cycle. A crucial role in both transitions is played by cyclin-dependent kinases (CDK), as first demonstrated in the fission yeast *Schizosaccharomyces pombe* (Nurse & Bissett, 1981). Later, it was shown that human cells contain homologs of the yeast Cdc2 CDK (Lee & Nurse, 1987). Cdc2 homologs were found also in plant cells, with their phosphorylation state being cell cycle-dependent (John *et al*, 1989). These studies paved the way for identifying Cdc2-like kinases (John *et al*, 1989; Feiler & Jacobs, 1990; Ferreira *et al*, 1991), as well as their A- and B-type cyclin partners (Hata *et al*, 1991; Hemerly *et al*, 1992; Hirt *et al*, 1992), in various plant species.

Identification of the CDK/cyclin targets controlling the G1/S transition proved to be a difficult task, which was enlightened by research in cancer. It was found that cells of rare human tumors, such as retinoblastoma or sarcoma, harbor inactivating mutations in the retinoblastoma susceptibility gene, RB1 (Friend *et al*, 1986; Fung *et al*, 1987; Lee *et al*, 1987). RB1 suppresses cell proliferation and can bind oncoviral proteins such as SV40 large T-antigen (T-ag; Lee *et al*, 1987; DeCaprio *et al*, 1988; Dyson *et al*, 1989; Ludlow *et al*, 1989). Moreover, RB1 was found to be phosphorylated in a cell cycle-dependent manner by CDKs (Lees *et al*, 1991). Together, these results led to the proposal that RB1 is a regulator of cell cycle progression in G1 (Buchkovich *et al*, 1989).

The last piece in the initial puzzle was the identification of E2F (for "adenovirus early gene 2 promoter-binding factor") as a cellular protein that could form complexes with RB1 (Chellappan *et al*, 1991). These and other findings served to establish that the RB1 complexes are regulators of the G1/S transition, as well as of the switch from quiescence to proliferation (reviewed in Fischer & Müller, 2017). Other RB1 interactors, primarily D-type cyclins, bind RB1 through a LxCxE amino acid motif, an interaction that is mimicked by human oncoviral proteins that inactivate RB1's tumor suppressor function (DeCaprio, 2009); however, although the finding of the LxCxE motif in D-type cyclins proved originally valuable, more recent work deleting this motif in mammalian cells has demonstrated that it is not essential (Landis *et al*, 2007; Topacio *et al*, 2019).

Retinoblastoma protein in plants

Back in the mid-1990s, a couple of apparently unrelated research lines reinforced the hypothesis that plants might share some kind of RB/E2F regulatory module with human cells. D-type cyclin

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homologs were identified in several plant species (Dahl *et al*, 1995; Soni *et al*, 1995), which had—despite limited amino acid sequence homology with human D-type cyclins—two key features in common with them: a similar expression pattern during the cell cycle and a highly conserved LxCxE amino acid motif (Soni *et al*, 1995; Riou-Khamlichi *et al*, 1999). These facts strongly suggested that plants might contain proteins that could recognize this motif, as it is the case for human cyclin D and RB1. Independently, geminiviruses, a group of plant DNA viruses that replicate their single-stranded DNA genome in the nucleus of the infected cell, were found to trigger synthesis of the host cell nuclear DNA upon infection (Nagar *et al*, 1995). Additionally, wheat dwarf virus (WDV) and other monocotyledonous plant-infecting geminiviruses were found to encode an LxCxE motif-containing protein, RepA (Xie *et al*, 1995). Importantly, this motif required for efficient viral DNA replication was shown to enable interaction of RepA also with human RB1 (Xie *et al*, 1995), in a manner analogous to human oncoviral proteins. Subsequent studies identified genes encoding retinoblastoma-related (RBR1) proteins in maize (Grafi *et al*, 1996; Xie *et al*, 1996; Ach *et al*, 1997a), and in an immediate follow-up to the cloning of multiple plant E2F and DP factors (Ramirez-Parra *et al*, 1999; Sekine *et al*, 1999; Albani *et al*, 2000; Magyar *et al*, 2000; Ramirez-Parra & Gutierrez, 2000; Kosugi & Ohashi, 2002). Remarkably, Arabidopsis was found to encode six E2F family members with two types of domain organization. The first group (E2FA, B and C) possesses the same domains as human E2F1-5, including DNA-binding and dimerization domains. The second group (E2FD, E and F, also known as DEL2, 1 and 3, respectively) is unique in that its members contain a duplicated DNA-binding domain but lack the dimerization domain that allows binding to DNA in the absence of DP factors and fails to activate gene expression (Kosugi & Ohashi, 2002; Mariconti *et al*, 2002). Proteins homologous to these atypical E2F family members were subsequently also identified in mammalian cells (reviewed in Trimarchi & Lees, 2002; Lammens *et al*, 2009). Genome-wide studies identified putative E2F target genes in the Arabidopsis genome (Ramirez-Parra *et al*, 2003; Vandepoele *et al*, 2005; Naouar *et al*, 2009), a list that—somewhat surprisingly at the time—contained not only *bona fide* cell cycle control genes, but also genes involved in many other aspects of plant physiology, strongly pointing to a multifunctional role of RBR1. This will be further discussed below.

Evolutionary perspective on plant RBR proteins

The availability of multiple plant genomes has revealed the presence of RBR-, E2F-, and DP-encoding genes in all species analyzed so far (reviewed in detail in Gutzat *et al*, 2014; Desvoyes *et al*, 2014). A first conclusion from the genomic data is that the appearance of the RBR-E2F/DP module preceded the multiple branches of multicellularity that occurred ~800 million years ago, since it is present in unicellular organisms such as *Chlamydomonas reinhardtii* (Umen & Goodenough, 2001) and *Ostreococcus tauri* (Robbens *et al*, 2005), as well as colonial algae such as *Volvox carteri* (Kianianmomeni *et al*, 2008; Fig 1). Within the multicellular plant lineages, RBR proteins are present in all of them, and notably, monocotyledonous plants possess several members with different functions (Fig 1; see discussion below); for a more detailed discussion on how the RBR-E2F/DP module has evolved in plants and animals, please refer to (Desvoyes *et al*, 2014). It is likely that RBR and other family members may have evolved specific functions in the different plant lineages. Thus, contrary to dicotyledonous plants that encode for a single RBR1 protein, monocotyledonous plants such as maize or rice contain two major RBR subfamilies: one whose members are involved in negative regulation of cell cycle, e.g., maize RBR1 and RBR2, and another involved in endosperm development, e.g., maize RBR3 and RBR4 (Sabelli *et al*, 2005; Sabelli & Larkins, 2006; Lendvai *et al*, 2007).

The current data are consistent with the idea that the RBR-E2F/DP module is an ancient invention likely present already in the last eukaryotic common ancestor (LECA; Desvoyes *et al*, 2014). Later, the RBR/E2F-DP module has been lost in some plant lineages, e.g., *Ulvophyceae* (De Clerck *et al*, 2018), and in other eukaryotes, e.g., *S. cerevisiae* and other yeast (Desvoyes *et al*, 2014). It is likely that organisms lacking RBR or related proteins use different pathways to regulate G1 progression. In *S. cerevisiae*, the Whi5 protein (Jorgensen *et al*, 2002), which is not homologous to animal RB1 or plant RBR1, seems to play analogous functions, whereby increased Cdk activity associated with the G1 cyclin Cln3 mediates activation of the G1 transcription factor heterodimer Swi4/Swi6, also known as SBF. Like RB proteins, Whi5 negatively regulates SBF, and its phosphorylation by Cdk/Cln3 complexes frees SBF to activate its targets (de Bruin *et al*, 2004; Costanzo *et al*, 2004). Together, these evolutionary studies reinforce the usefulness of comparative research on key cellular pathways.

Plant RBR1 domains and phosphorylation states

As expected from their ancient evolutionary origin, plant RBR proteins share with animal counterparts not only a relatively high degree of amino acid homology but, more importantly, a similar organization of functional domains. This includes an N-terminal domain, the central A/B domain that forms the "pocket," and a C-terminal domain (Fig 2). A groove in the B domain is necessary for binding of LxCxE-containing proteins, and together with the C-terminal domain mediates interaction with E2F (Rubin *et al*, 2005). In addition, intrinsically disordered regions are present between the A and B domains as well as in the majority of the C-terminal domain. It is remarkable that these regions contain most of the conserved CDK phosphorylation sites, including T373, involved in driving a more globular structure of this region, and S608, whose phosphorylation prevents interaction with E2F, as described for human RB1 (Burke *et al*, 2010).

Plant RBR proteins contain putative CDK phosphorylation sites in similar locations, although the role of individual sites on RBR structure and function is not yet known. In spite of this, there are reports on the overall function of RBR1 phosphorylation in cell cycle control in various plant species. The mechanism mediating RBR1 activity through phosphorylation involves the interaction with CDK/cyclin complexes containing many plant D-type cyclins (Grafi *et al*, 1996; Nakagami *et al*, 1999, 2002; Boniotti & Gutierrez, 2001; Gutiérrez *et al*, 2005; Godínez-Palma *et al*, 2017) and either CDKA or CDKB types of kinases (Boniotti & Gutierrez, 2001; Kawamura *et al*, 2006). The CDK/cyclin activity on RBR1 is cell cycle-regulated and highest from mid-G1 phase until the G1/S transition (Boniotti & Gutierrez, 2001; Nakagami *et al*, 2002; Sanchez *et al*, 2002; Hirano

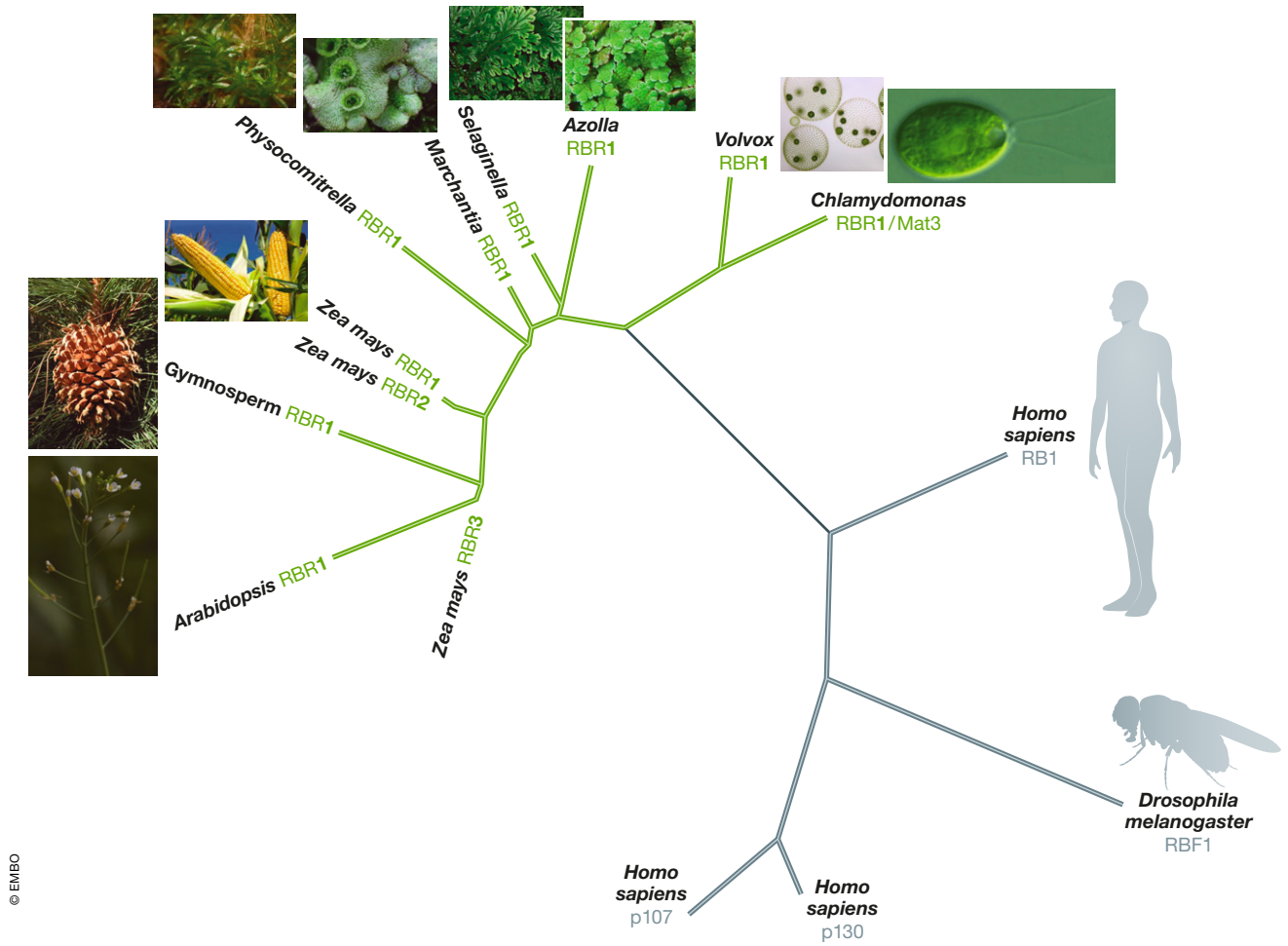

Figure 1. Phylogenetic relationships of RB family members from representative animal and plant lineages.

Homo sapiens (Human, mammal); *Drosophila melanogaster* (Artropoda); *Chlamydomonas reinhardtii* (Algae, unicellular); *Volvox carteri* (Algae, colonial); *Azolla filliculoides* (Fern); *Selaginella moellendorffii* (Lycophyte); *Marchantia polymorpha* (Liverwort); *Physcomytrella patents* (Moss); *Pinus silvestris* (Gymnosperm); *Arabidopsis thaliana* (Angiosperm, dicotyledonous); *Zea mays* (Angiosperm, monocotyledonous).

et al, 2008; Nowack *et al*, 2012), as well as and in G2 mediated by CDKB1;1 (Kawamura *et al*, 2006; Nowack *et al*, 2012). These CDK activities can be suppressed by CDK inhibitors known as Kip-related proteins (KRPs; Pettkó-Szandtner *et al*, 2006). In unicellular algae, RBR1/CDK/cyclin ternary complexes remain bound to chromatin throughout the cell cycle (Olson *et al*, 2010), but this has not been corroborated in multicellular plants. A functional connection between members of the RBR-E2F/DP-CDK/cyclin module has been demonstrated by the finding that activation of PCNA expression by E2F/DP is inhibited by co-expression of RBR1, an inhibition counteracted by additional expression of cyclin D (Uemukai *et al*, 2005; Shimizu-Sato *et al*, 2008).

Antibodies detecting specific phosphorylated residues in human RB1, such as pS807/pS811, proved useful to identify phosphorylated forms of RBR1 that accumulate after the G1/S transition and, in particular, during the G2 phase (Abrahám *et al*, 2011; Polit *et al*, 2012). It is interesting that phosphorylated RBR1 can be detected in the nucleus of interphase cells in the form of granules (Abrahám *et al*, 2011), although the functional relevance of such foci remains to be determined. Proteomic studies have demonstrated RBR1 phosphorylation *in vivo* at residues T406, S652, and S911 (Reiland *et al*, 2009; Willems *et al*, 2020). The identification of other phosphorylated residues, as well as their functions, remains as a future challenge. RBR1 phosphorylation is important not only for cell cycle progression but also for cell fate determination, as exemplified in the stem cell niche of the root apical meristem (RAM; Cruz-Ramirez *et al*, 2012; see discussion below). Plant RBR1 is also a substrate of other kinases, e.g., PIP5K (Dieck *et al*, 2012) or S6K (Henriques *et al*, 2010, 2013), but in these cases the mechanistic implications are not fully understood.

RBR in unicellular green organisms

The lack of RB1 orthologs in yeasts and their presence in animals and plants had initially suggested that it may have been an evolutionary acquisition linked to multicellularity. This hypothesis was however ruled out by the identification of RBR1 homologs as well

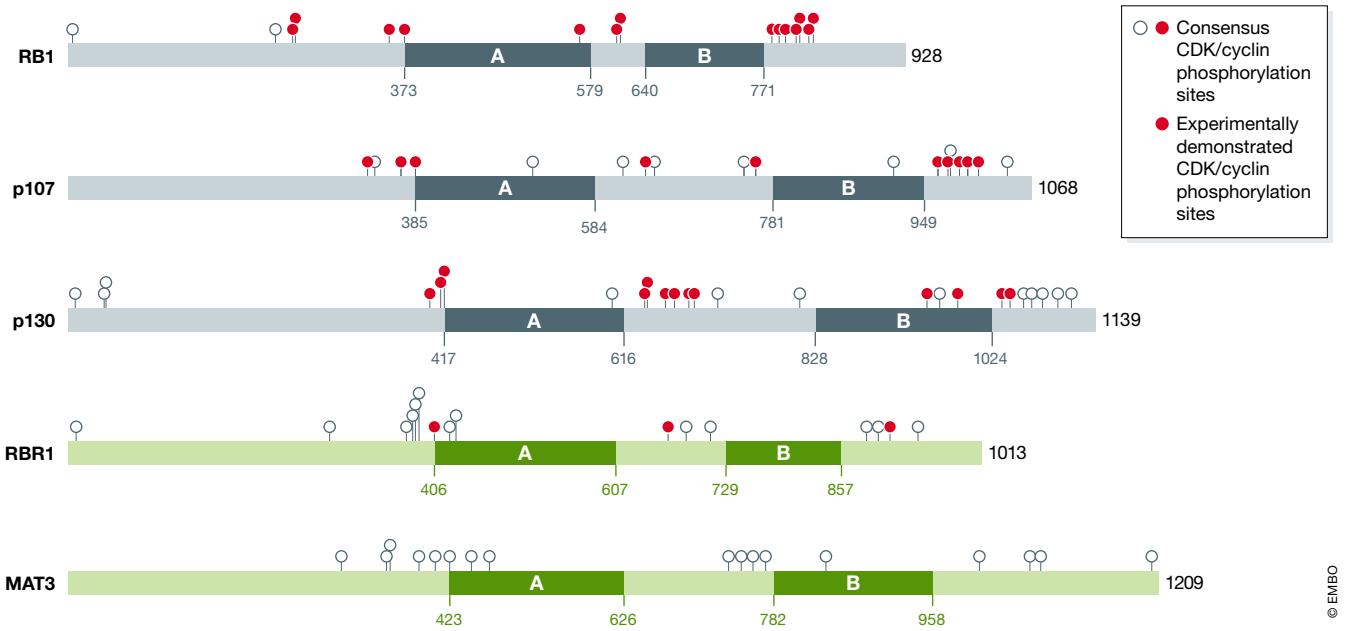

Figure 2. Domain organization of representative proteins of the RB family.
Domain organization of retinoblastoma family proteins comparing the three human proteins (RB1, p107, and p130) with two plant retinoblastoma-related proteins (*Arabidopsis thaliana* RBR1 and *Chlamydomonas reinhardtii* MAT3). The two major domains A and B (green) defining the "pocket" together with the amino acid positions are indicated. The CDK/cyclin consensus phosphorylation sites (empty circles) are also indicated. Those experimentally demonstrated to be phosphorylated are shown (closed red circles). Data are taken from the UNIPROT database (Hansen *et al*, 2001; Farkas *et al*, 2002; Rubin, 2013; Willems *et al*, 2020).

as its E2F/DP partners in the unicellular alga *Chlamydomonas reinhardtii* (Umen & Goodenough, 2001; Fang *et al*, 2006). This organism operates a peculiar cell division cycle, since a mother cell grows in G1 to much more than twice its size, and upon reaching a critical size then undergoes multiple cycles of S-phases and mitoses to produce multiple daughter cells of the size typical for this organism. However, RBR1 does not regulate the length of G1, but instead acts to counter the number of mitotic divisions, a process that further depends on E2F/DP (Umen & Goodenough, 2001; Fang *et al*, 2006), a SUMO peptidase (Fang & Umen, 2008), and a unique G-type CDK (Li *et al*, 2016). Remarkably, periodic expression of cell cycle genes is independent of functional RBR, E2F, and DP in this unicellular alga (Fang *et al*, 2006), altogether suggesting that an early role in evolution of RBR proteins was likely related to cell size control, rather than cell cycle progression through G1.

Non-cell cycle functions of RBR proteins are also found in the colonial alga *Volvox carteri*, where the RBR1 gene has a gender-specific expression pattern in female cells (Kianianmomeni *et al*, 2008). In these cells, up to four differentially spliced products of the RBR1 gene have been identified (Kianianmomeni *et al*, 2008; Hallmann, 2009; Ferris *et al*, 2010), suggesting a multifunctional role of RBR1 in different processes that still need to be delineated.

Transcriptional waves controlled by RBR1 during the cell cycle

As in animal cells, two major transcriptional waves can be distinguished in plants during G1 and G2 phases, which regulate expression of gene products required for S-phase and mitotic progression, respectively.

RBR1-E2F complexes

In the case of G1, several lines of evidence demonstrate that the key role of the RBR1-E2F module includes the negative RBR1 regulation by CDK/cyclin complexes, the counteracting CDK inhibitors (KRPs for Kip-related proteins), and the participation of chromatin remodeling enzymes, such as histone acetyl transferases (HATs), and subsequent recruitment of RNA polymerase II at the target promoters (Fischer & Müller, 2017). High levels of E2FA/DPA or E2FC/DPB expression lead to misregulation of E2F target genes and developmental abnormalities in *Arabidopsis* (De Veylder *et al*, 2002; del Pozo *et al*, 2002, 2006). Modest overexpression of E2FA or CYCD3 directly affects expression of their target genes and demonstrated that E2FC does not counteract E2FA-mediated gene upregulation (see also discussion of E2FC roles in G2, below). Furthermore, cell wall biogenesis depends on E2FA (de Jager *et al*, 2009) and on E2FF/DEL3 (Ramirez-Parra & Gutierrez, 2007). Direct RBR1 regulation by CDKA;1 was shown using *cdka;1* mutants able to rescue the *rbr1* mutant phenotype (Nowack *et al*, 2012). However, RBR1 is not exclusively regulated by CDKA;1, and S6K was found as another RBR1-interacting kinase phosphorylating it and repressing cell proliferation by inhibiting E2FB factors (Henriques *et al*, 2010, 2013). Interestingly, RBR1 can form a stable repressor complex with E2FA but not with E2FB. In the proliferation area, differentiation genes such as *CCS52A1* and *CCS52A2* (encoding activators of the anaphase promoting complex/cyclosome (APC/C)), are repressed under conditions of high cyclin D/CDK activity (Magyar *et al*, 2012).

Other upstream regulators included the KRP family of CDK inhibitors, formed by seven members in *Arabidopsis*, and the F-box-like protein FBL17 (Kim *et al*, 2008; Zhao *et al*, 2012; Noir *et al*, 2015). FBL17 is itself regulated by the RBR1/E2F pathway and at the same time (as subunit of an SCF ubiquitin ligase) mediates degradation of several downstream targets of E2F, e.g., CDT1a (Desvoyes *et al*, 2019). CDK activity is also inhibited by another family of proteins, SIAMESE (SIM) and SIAMESE-RELATED (SMR), and some SMRs are under control of the TARGET OF RAPAMYCIN (TOR) signaling pathway (Ahmad *et al*, 2019; Barrada *et al*, 2019). Therefore, RBR1-mediated gene expression in G1 is controlled by multiple redundant pathways including kinases, inhibitors, and ubiquitin/proteasome-dependent degradation, to finely balance the availability of gene products required for G1/S transition and S-phase progression (Fig 3A and B).

DREAM complexes

RB family proteins form parts of multimeric complexes that coordinate transcriptional waves during the cell cycle, originally identified as dREAM in flies (for *Drosophila* RBF, E2f2 and Multi-vulval interacting proteins; Lewis *et al*, 2004), DREAM in mammals (Litovchick *et al*, 2007), DRM in *C. elegans* (Harrison *et al*, 2006), and DREAM-like in plants (Kobayashi *et al*, 2015).

In mammalian cells, DREAM complexes are master regulators of the cell cycle that repress gene expression in quiescent cells and in G1 with the participation of the RB-related pocket proteins p130 and p107 (while RB1 itself is restricted to repressing E2F targets) together with the MuvB core components (LIN-9, LIN-37, LIN-52, LIN-54 and RBBP4). DREAM regulates gene repression in mammalian G1 phase by two different modes, one dependent on repressor E2F4/5 binding to E2F sites and another on the presence of the DREAM core component LIN-54 that recognizes a DNA sequence motif called "cell cycle gene homology region" (CHR), also present in the promoters of many G2/M genes. In S-G2 phase, a different Muv-Myb complex then forms to regulate genes required for mitosis, a complex assembled by subsequent recruitment of B-MYB and the forkhead transcription factor FOXM1 (Sadasivam & DeCaprio, 2013; Fischer & Müller, 2017).

In contrast, the Arabidopsis DREAM complex contains both MYB transcription factors and RBR1 alongside orthologues of the core DREAM elements (Fig 3A and B), as shown by mass spectrometry experiments (Kobayashi *et al*, 2015; Fischer & Müller, 2017; Horvath *et al*, 2017): ALY2 and ALY3 (ALWAYS EARLY proteins) are orthologues of LIN-9, TCX5 (Tesmin/TSO1-like CXC domain protein) is orthologous to LIN54, and MSI1 (MULTI-COPY SUPRESSOR OF IRA1) is orthologous to RBBP4. While orthologues of LIN-37 and LIN-52 have not been found in *Arabidopsis*, E2FC/B, DPA/B, CDKA (suggestive of phosphorylation as a potential regulator of DREAM activity), and MYB proteins were all found associated with plant DREAM. *Arabidopsis* contains multiple *MYB3R* genes (Kobayashi *et al*, 2015), with *MYB3R1 and MYB3R4* regulating the expression of *CYCLIN B* or *KNOLLE* (Haga *et al*, 2007, 2011). The promoters of these G2/M genes contain the MSA (Mitosis-specific activator) motif, originally identified in tobacco BY-2 cells (Ito *et al*, 1998). Analysis of *Arabidopsis myb3r3*, *myb3r5* double mutants (*myb3R3/5*) showed activation of G2/M genes both in proliferative and mature tissues, supporting a role of *Arabidopsis* DREAM in gene expression control in G2. A triple *myb3R1/3/5* mutant exhibits

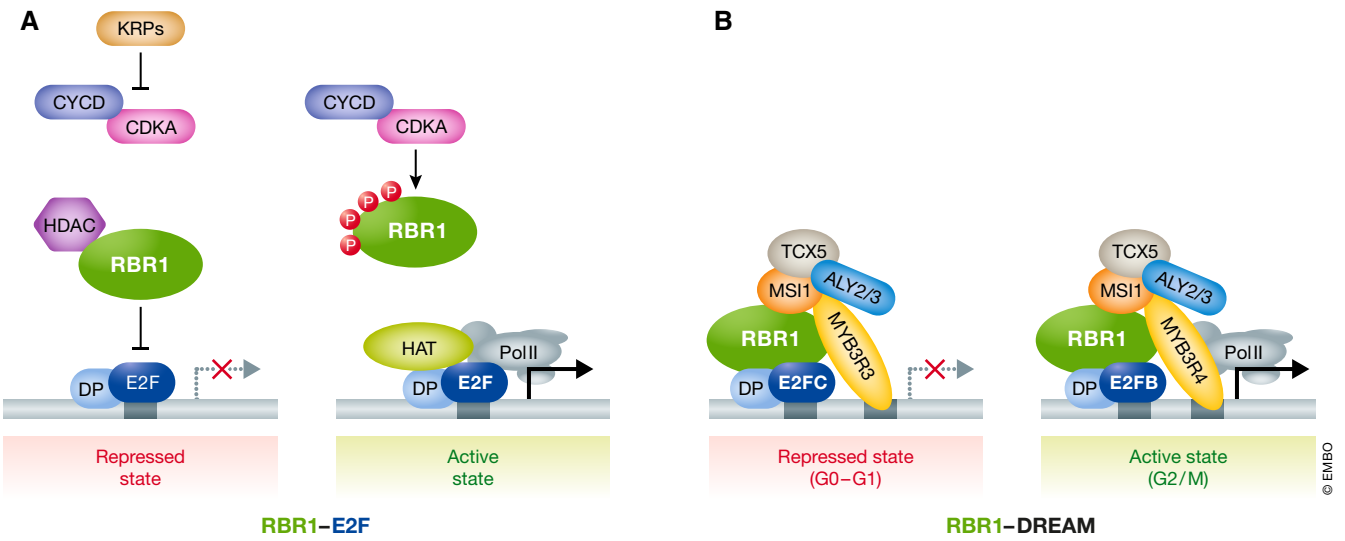

Figure 3. Role of Arabidopsis RBR1 complexes in transcriptional control during the cell cycle.

(A) RBR1-E2F complexes. Genes required for the G1/S transition are bound by E2F-DP heterodimers located at the E2F binding sites in their promoters (grey box). They are repressed by the retinoblastoma-related (RBR1) protein in association with histone deacetylases (HDAC). At this stage, CDKA-cyclin complexes are inactivated by one or more CDK inhibitors (KRPs). In Arabidopsis, E2FA-C, bound to DP partners, participate in regulation of different gene targets. Later in G1, when CYCD levels are sufficiently high, CDKA phosphorylates RBR1 (small red circles) leaving free the E2F-DP complexes to transactivate their target genes, in association with histone acetylases (HAT) after recruitment of RNA polymerase II (Pol II). (B) RBR1-DREAM complexes. RBR1 also participates in other transcriptional regulatory complexes. Briefly, at the repressed state, while CDK are inactive, E2FC is part of the complex together with the repressor MYB3R3 factor and RBR1. E2F and MYB factors bind to different sites in the promoter of target genes (white boxes). The DREAM complex switches to an activator when E2FB and MYB3R4 factors are incorporated (see text for details on composition and function).

enlarged organs resulting from increased cell proliferation, revealing that MYB3R3/5 are repressors while MYB3R1 has a dual activator and repressor role (Kobayashi *et al*, 2015).

Chromatin immunoprecipitation of MYB3R3 has revealed promoters of early cell cycle and mitotic genes that contain E2F and MSA binding sites, respectively, hinting to the existence of distinct DREAM complexes (Kobayashi *et al*, 2015). In fact, binding sites of RBR1 and MYB3R3 mostly coincide at promoter regions of S-phase- and G2/M-regulated genes (Bouyer *et al*, 2018). Furthermore, RBR1, DP, and either repressors E2FC and MYB3R3 or activators E2FB and MYB3R4 are present in the two different complexes acting in G1 and in G2/M, respectively, a situation different from the case of animal cells (Fischer & Müller, 2017). In this regard, it is worth noting that in contrast to animal cells, plant cell G2 phase requires expression of multiple genes regulated by E2F, including CDKB-type kinases (Boudolf *et al*, 2004). Additional components of the DREAM complex have been identified by genetic interactions. For example, loss-of-function mutations in the *TSO1* gene (Andersen *et al*, 2007), causing overproliferation of meristems and defects in flower development, are suppressed by *myb3r1* mutation but not by *myb3r4* mutation, although no direct TSO1 interaction with RBR1 was found (Wang *et al*, 2018).

DREAM target genes appear to extend beyond cell cycle genes. In a recent study, TCX5 was found to repress the expression of *MET1*, *CMT3*, *DDM1*, *KYP*, and *VIMs* genes involved in maintenance of DNA methylation (Ning *et al*, 2020). TCX5 is redundant with its paralogue TCX6, and the double mutant *tcx5, tcx6* exhibits increased levels of DNA methylation, primarily at CHG sites. Another recent study identified that SOL1/TCX3 and SOL2/TCX2, two SPEECHLESS targets from the *TSO1*-like family, are important regulators of fate transition in the stomatal lineage (see also discussion on RBR1 role in cell fate acquisition in the stomatal lineage). It was hypothesized that they could compete with DREAM for binding sites on DNA (Simmons *et al*, 2019), but direct evidence for the participation of TSO1/MYB3R1 and SOL1/SOL2 in the DREAM complex is still lacking.

Interplay of RBR1 with chromatin

Early studies in plants, parallel to those in animals, already showed a connection of RBR1 with chromatin components and provided insightful information about its potential functional relevance. Plant RBR1 was found to interact with MSI1, a homolog of human RbAp46/48 (Ach *et al*, 1997b; Lusser *et al*, 1999; Rossi *et al*, 2001), and with the histone deacetylase (HDAC) Rpd3 for repression of target gene expression (Rossi *et al*, 2003).

Independent studies of flowering control and response to cold stress identified MSI4 (encoded by the *FVE* gene), a protein that interacts not only with RBR1 but also with HDACs (Ausin *et al*, 2004; Kim *et al*, 2004; Pazhouhandeh *et al*, 2011). Interestingly, *fve* mutants show increased histone acetylation levels and abnormal silencing of transposable elements (the latter process also affected by RBR1), through effects on cytosine methylation (mC) at CHH and CHG sites (Gu *et al*, 2011; Xu *et al*, 2013). Participation in controlling mC levels implies a role in imprinting, which in plants relies on removing silencing marks. Consistent with this, RBR1 interacts with the mC demethylase DEMETER (DME) and represses MET1 (METHYLTRANSFERASE 1), an E2F target gene (Jullien *et al*, 2008).

Further support for a role of RBR1 in repressing euchromatic genes and in TE silencing comes from more recent genome-wide mapping of RBR1 binding sites, which colocalize largely with previously identified E2F and MYB3R3 target genes (see also discussion on DREAM; Bouyer *et al*, 2018). It is worth noting that TEs have amplified E2F binding sites, as revealed by the presence of ~85% of all E2F binding sites in the heterochromatin of *Arabidopsis* and other *Brassicaceae* (Henaff *et al*, 2014).

In addition to the interaction of RBR1 with factors involved in regulating histone acetylation levels, RBR1 also plays a role in maintenance of the repressed state of polycomb (PcG) chromatin. This relies on the physical interaction of RBR1 with PRC2 (polycomb-repressive complex 2) components such as FIE, CLF, and VNR2 (Mosquna *et al*, 2004; Guitton & Berger, 2005a; Johnston *et al*, 2010). Defects in RBR1-PRC2 interaction result in mutant phenotypes during *Arabidopsis* gametophyte development and during cell fate acquisition (Johnston *et al*, 2008, 2010), but elucidation of detailed mechanisms involved in the RBR1-PRC2 pathway awaits further research (reviewed in Kuwabara & Gruissem, 2014).

RBR1 in the DNA damage response (DDR)

Plant and animal DNA damage responses share several general strategies, but they also exhibit several unique features. In addition to the conserved ATM and ATR pathways, the plant DDR depends on transcriptional activation of plant-specific target genes such as SOG1, and on epigenetic modifiers (reviewed in Kim, 2019; Nisa *et al*, 2019). The first hint of the participation of RBR1 in DDR came from the observation that the typical nuclear foci marked by phosphorylated variant histone H2AX (γH2AX) formed after DNA damage contained E2F and depended on an intact RBR1-binding motif in E2F (Lang *et al*, 2012). Upon DNA damage, RBR1 and E2FA are recruited to γH2AX foci in an ATM- and ATR-dependent manner (Horvath *et al*, 2017). This recruitment process stimulated by the plant-specific CDKB1 not only involves BRCA1, which physically interacts with RBR1, but also the recombinase RAD51 (Biedermann *et al*, 2017; Horvath *et al*, 2017). Whether RBR1 serves as a landing pad or as a recruitment factor for DDR proteins that accumulate at sites of genomic DNA damage, or whether it plays additional roles, is not yet fully understood. Many DDR genes are regulated by RBR1 binding to their promoters. Combining transcriptome analysis after DNA damage and genome-wide analysis of RBR1 binding sites allowed identification of yet uncharacterized DDR genes, such as FIDGETIN-LIKE-1 INTERACTING PROTEIN (FLIP) involved in homologous recombination (Bouyer *et al*, 2018; Fernandes *et al*, 2018). Moreover, the DREAM complex also participates in the DDR, since repressor MYB3R3 and MYB3R5 suppress expression of a subset of G2/M genes required for cell cycle arrest after DNA damage (Chen *et al*, 2017). In mutants lacking FBL17, a major regulator of cell cycle progression and endoreplication, DDR genes are constitutively upregulated in the absence of DNA damage (Gentric *et al*, 2020). This FBL17-mediated DDR gene regulation is SOG1-independent, and recruitment of FBL17 to double-strand breaks (DSB) leads to its colocalization with γH2AX in a RBR1-dependent manner. Furthermore, since RBR1 colocalizes with either γH2AX or

FBL17 in about 5% of cases and the percentage of foci containing all the three proteins is even lower (~1%), it is likely that recruitment of RBR1 and FBL17 to DNA repair foci is a dynamic process (Gentric *et al*, 2020). The participation of RBR1 in DSB repair may share some mechanistic aspects with its requirement for the production of meiocytes (Zhao *et al*, 2017b). Furthermore, an *rbr1-2* allele-bearing mutant with reduced levels of RBR1, despite showing normal vegetative development, displays reduced chiasma formation during meiotic prophase I (Chen *et al*, 2011).

RBR1 in asymmetric division and stem cell maintenance

Stem cells are crucial elements in the maintenance of cellular homeostasis, as they ensure a continuous supply of pluripotent cells necessary for organogenesis as well as replenishment of damaged cells during regeneration processes. Stem cells divide asymmetrically at a relatively slow rate and give rise to one cell that remains a stem cell and another cell that initiates acquisition of a given fate while maintaining an active division rate. In the case of plants, where cell movement is precluded by the presence of a cell wall, stem cells remain confined within niches, in direct contact with an organizing center that provides the necessary signals to maintain their stemness nature. The best studied stem cell niches in the plant body are located in the shoot and root apical meristems (Sablowski, 2007; Scheres, 2007). Further cells with stem cell potential are dispersed throughout, responsible for the formation of the stomatal lineage, located in the leaf blade (Han & Torii, 2016; Lee & Bergmann, 2019), among others.

The RBR1-E2F module plays a crucial role in stem cell maintenance. Thus, reduction of RBR1 or increase of E2FA levels in the root apical meristem (RAM) likewise lead to an increase in stem cell number; *vice versa*, overexpression of RBR1 leads to a severe reduction of stem cell number (Wildwater *et al*, 2005). RBR1 is also required cell-autonomously to limit the division of quiescent center cells in the root meristem (Cruz-Ramirez *et al*, 2013) Furthermore, a *cdka;1* mutant, albeit viable, exhibits pleiotropic developmental abnormalities, demonstrating that CDKA;1 is part of a pathway contributing to stem cell maintenance by controlling the phosphorylation state of RBR1 (Nowack *et al*, 2012). In fact, the *rbr1-1* mutation can rescue the stem cell defects in the *cdka;1* mutant. The precise phospho-sites required for this RBR1 function remain to be determined.

The asymmetrical nature of stem cell divisions gives rise to two daughter cells that are frequently different in size, but more importantly, one of the daughters acquires a distinct cell fate. In some cases, the following formative divisions are also asymmetrical. In addition to specific transcription factors required for conferring stemness in various plant organs (De Smet & Beeckman, 2011), there is evidence that cell cycle factors act in a coordinated manner. Indeed, there are two examples where RBR1 is involved in the control of asymmetrical cell division (ACD) and terminal cell fate acquisition: formation of endodermis and cortex in the RAM (Wildwater *et al*, 2005; Bennett & Scheres, 2010) and in the stomatal lineage (Lee & Bergmann, 2019), respectively.

In the distal part of the RAM, the stem cell that gives rise to the endodermis and cortex cell layers, the so-called cortex/endodermis initial (CEI), first divides anticlinally and asymmetrically to produce one cell that remains a CEI, and another that becomes a cortex/endodermis initial derivative (CEID). This then divides periclinally and again asymmetrically to form the initials of the endodermis and the cortex cell lineages (Fig 4A and B). SCARECROW (SCR) and SHORTROOT (SHR), two members of the GRAS family of transcription factors, are crucial for the maintenance of the stem cell nature of the CEI (Sabatini *et al*, 2003). An SHR/SCR heterodimer activates expression of various target genes, one of them being *CYCLIND6;1* (*CYCD6;1*), specifically expressed in the CEID (Sozzani *et al*, 2010; Cruz-Ramirez *et al*, 2012). RBR1 interacts with SHR/SCR through the LxCxE motif of SCR, and RBR1 phosphorylation by CYCD6;1-CDK releases the transcriptional activity of SHR/SCR that triggers ACD (Fig 4A and B). RBR1 inactivation by phosphorylation likely occurs by a sequential activity of CDKB1;1 and CDKA;1, as revealed by the phenotypes of the corresponding mutants (Cruz-Ramirez *et al*, 2012; Weimer *et al*, 2012). CYCA3;4 is another CDK partner controlling formative divisions in connection to RBR1 phosphorylation, as demonstrated by the abnormal root meristem and stomatal lineage pattern in the presence of high levels of this cyclin (Willems *et al*, 2020). Once ACD has been successfully completed, daughter cells acquire endodermal or cortex fate depending on the presence or absence of SCR, respectively. An extra layer of regulation that resets the regulatory circuit just before mitosis comes with the proteasome-mediated degradation of at least SCR and RBR1 (Cruz-Ramirez *et al*, 2012). In addition, MED31, a subunit of the Mediator complex, also regulates CYCD6;1 in a SCR/SHR-dependent manner (Zhang *et al*, 2018), revealing the participation of different mechanisms to ensure proper ACD. However, the identity of certain cell types in the RAM (e.g., columella stem cells) not only depends on RBR1 but also on other regulatory networks, e.g. specific transcription factors such as SOMBRERO (SMB), FEZ, and auxin response factors that function in parallel (Bennett *et al*, 2014), revealing a large and still incompletely understood complexity, in the RAM stem cell niche. The participation of multiple RBR1-independent pathways is reinforced by the demonstration that the topoisomerase TOP1a is required for stele stem cell renewal (Zhang *et al*, 2016), in a manner modulated by ERF115, originally described as a factor required for controlling division of quiescent center cells (Heyman *et al*, 2013).

Early studies with RBR1 downregulation showed the need for functional RBR1 levels for proper development of leaf epidermal cells, including the stomatal lineage (Park *et al*, 2005; Desvoyes *et al*, 2006; Borghi *et al*, 2010). Loss of RBR1 function causes overproliferation of cells expressing stomata lineage markers in the leaf epidermis at different developmental stages, consistent with the occurrence of an amplification phase of meristemoids (Fig 4A and B). Moreover, increased expression of E2F targets, e.g., the DNA replication initiation proteins CDT1a or CDC6, leads to increased proliferation of meristemoid cells (Castellano *et al*, 2001, 2004), underscoring the role of the RBR1-E2F module in regulating cell division potential in the leaf epidermis.

In addition, formative divisions and cell fate acquisition in the stomatal lineage that give rise to mature stomata require the concerted action of various transcription factors and RBR1 (reviewed in Han & Torii, 2016). Protodermal cells in the epidermis of leaf primordia, the meristemoid mother cells (MMC), accumulate SPEECHLESS (SPCH), a substrate of various MAP kinases and of the protein phosphatase PP2A that together regulate SPCH stability (Bian

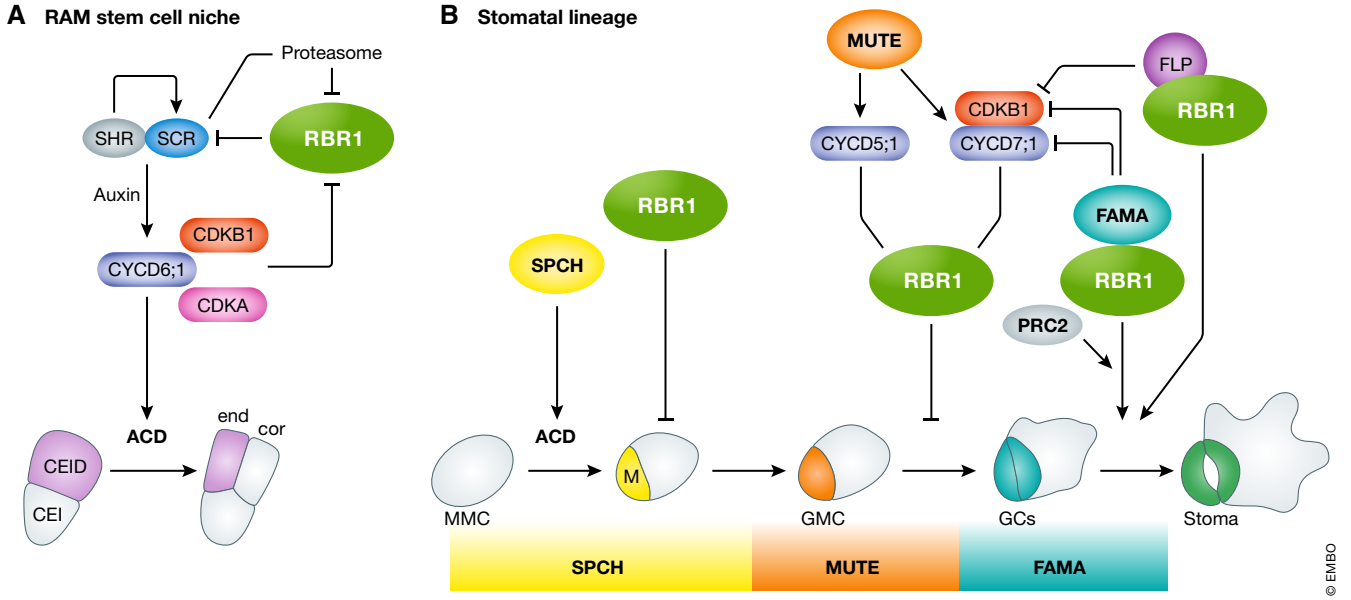

Figure 4. Role of Arabidopsis RBR1 in stem cell maintenance and asymmetric cell division.

(A) In the stem cell niche of the RAM, the cortex–endodermis initial derivative (CEID) cell undergoes an asymmetric cell division (ACD) that renders the initials of endodermis and cortex already having different fates. This ACD depends on CDK/CYCD6;1, the latter a target of the SHR/SCR heterodimer. The negative regulation of SCR by RBR1 is counteracted by RBR1 phosphorylation. (B) In the stomatal lineage, RBR1 also plays multiple roles including restriction of excessive proliferation of stem cells and the symmetric division of GMC to produce the young guard cells (GCs) as well as the terminal differentiation of GCs into mature guard cells of the stoma. This occurs by association with FAMA and polycomb repressor complex 2 (PRC2). The participation of FLP-RBR1 in terminal differentiation of GCs is also shown. The three major transcription factors acting in the stomatal lineage, SPCH, MUTE, and FAMA, are shown highlighting their functional windows.

et al, 2020). SPCH triggers asymmetrical cell division in MMCs, giving rise to a pavement cell and an immature meristemoid (M). At this proliferation stage, RBR1 restricts excess divisions of meristemoids, terminating their stem cell activity by the action of MUTE and leading to differentiation into a guard mother cell (GMC). A single symmetrical division of the guard mother cell, in which RBR1 plays its canonical role as a repressor of the G1/S transition, produces two young guard cells (GC). MUTE promotes *CYCD7;1* and *CYCD5;1* expression in a narrow window to release RBR1 repression by phosphorylation and, importantly, to assure that GMCs undergo only one cell division (Han *et al*, 2018; Weimer *et al*, 2018). It is worth noting that this division is also regulated by SOL1/TCX3 and SOL2/TCX2 (Simmons *et al*, 2019), members of the CHC family of proteins and putative members of DREAM complexes. The direct involvement of RBR1, SOL1, and SOL2 in a DREAM complex has not yet been experimentally confirmed. Finally, FAMA and FOUR LIPS transcription factors are redundantly required for the final differentiation of stomata guard cells, by inhibiting in an RBR1-mediated manner the expression of the E2F target CDKB1;1, necessary for the symmetric division of GMC (Boudolf *et al*, 2004; Lee *et al*, 2014). The FAMA-RBR1 complex, mediated by the LxCxE motif of FAMA, represses SPCH expression at late stages of stomatal development. The FAMA-RBR1 interaction is required for full differentiation of GC and maintenance of their cell fate, a process dependent on the PRC2 complex (Hachez *et al*, 2011; Matos *et al*, 2014). Downregulation of RBR1 in stomata and expression of an LxCxE-mutated FAMA unable to bind RBR1 reactivates SPCH expression in mature stomata and induces ACDs to form a stoma-in-stoma phenotype (Lee *et al*, 2014; Matos *et al*, 2014). Therefore, RBR1 is important for regulation of both cell cycle and cell fate genes to ensure the correct development of the stomatal lineage.

Cell division and differentiation balance during development

We have discussed various mechanisms and pathways in which RBR1 plays a crucial role at the cellular level. These are mainly related to cell cycle control by virtue of its ability to regulate gene expression at the transcriptional level and via its interaction with chromatin remodeling factors. As a consequence of these activities, RBR1 has also fundamental implications at the developmental and organismal level (Harashima & Sugimoto, 2016). The life cycle of plants, exemplified here by *Arabidopsis thaliana* (Fig 5), is defined by two main phases. The gametophytic phase consists of production of gametes, double fertilization leading to embryo and endosperm, and development of seeds. The sporophytic phase covers most of plant's life and consists of (i) vegetative growth occurring after seed germination, where most organs are formed post-embryonically, and (ii) the reproductive phase, when developmental and hormonal signals lead to the formation of flowers where gametes are formed (Fig 5). RBR1 is involved at virtually every stage and is crucial for the developmental phase transitions occurring during the entire life cycle.

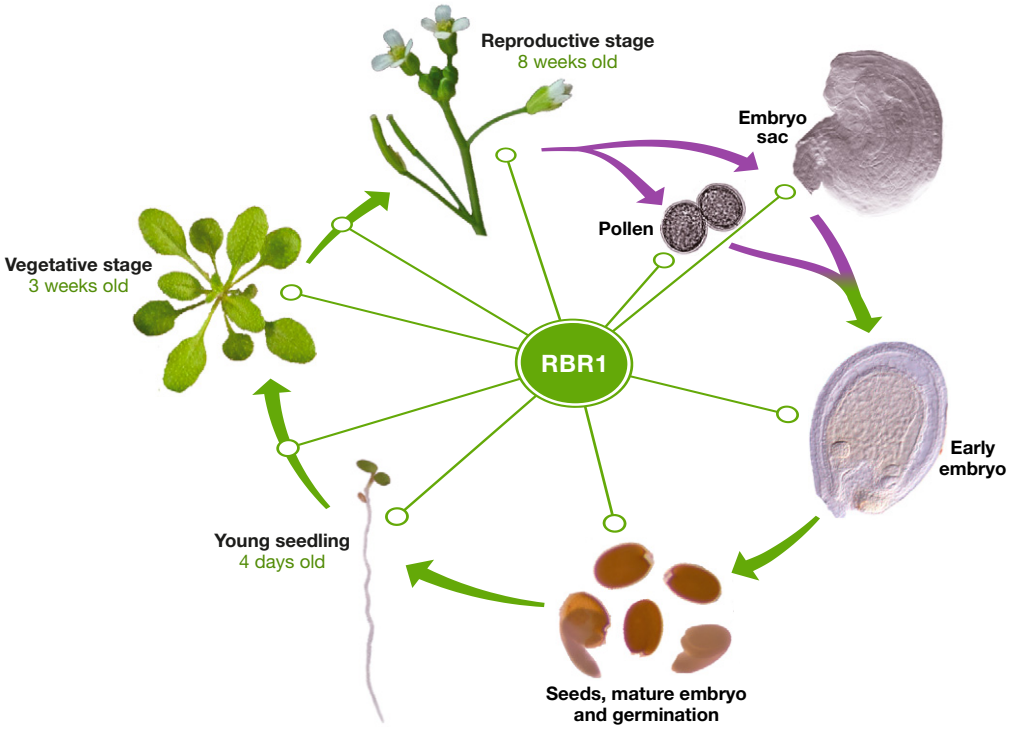

Figure 5. Role of RBR1 during the *Arabidopsis* life cycle.

Fully developed embryos present in the seeds grow after germination to produce young seedlings containing cotyledons as well as root and shoot apical meristems responsible for the growth of the root system and the aerial part, respectively. Rosette leaves appear later during vegetative growth. After the transition to the reproductive stage, flowers are produced where some cells specialize into precursors of germinal cells that produce haploid cells after meiosis. These haploid cells divide 2 or 3 times to produce the male and female gametophytes (mature pollen and embryo sac), respectively. After double fertilization, an embryo and the surrounding endosperm are formed inside the seed. Green and violet arrows denote the sporophytic and gametophytic phases, respectively. Most of these developmental stages and transitions are affected by RBR1 (see the text for details).

In the female part, most plants develop gametophytes through three consecutive cell divisions of the haploid products of meiosis, leading to two synergids, one egg cell, one central cell, and three antipodal cells. Here, RBR1 is required to restrict the number of these divisions (Ebel *et al*, 2004). Embryo formation upon fertilization of the ovule involves rapid cell divisions that are regulated by CYCD7;1 (Sornay *et al*, 2015), likely through control of RBR1 phosphorylation. At this stage, genes required later during seed maturation, such as *LEC1*, *LEC2*, *ABI3*, or *FUSCA*, are repressed by E2FA and E2FB, as demonstrated by their upregulation in a *e2fa, e2fb* double mutant (Leviczky *et al*, 2019). Surrounding the embryo, endosperm develops after fertilization of the central cell nucleus in a process that is restricted by the interaction of RBR1 with FIE (Mosquna *et al*, 2004). Furthermore, the possibility of parthenogenetic development of the embryo is restricted by RBR1 through its interaction with MSI1 (Guitton & Berger, 2005a,b). Since RBR1 is expressed across the entire female gametophyte (Ingouff *et al*, 2006), mechanisms must exist that control RBR1 function in different locations. In monocotyledonous plants, where the endosperm is quantitatively very important, an initial phase of cell proliferation when RBR1 represses the monocot-specific family member RBR3, precedes the switch to the endoreplication phase (Sabelli *et al*, 2005; Sabelli & Larkins, 2009). Expression of wheat dwarf virus RepA protein, which blocks RBR1 activity, can up-regulate RBR3, but not RBR1 (Sabelli *et al*, 2005). Later, RBR1 is required for repression of the proliferation phase and for promoting the endoreplication phase of endosperm development (Sabelli *et al*, 2013).

After seed germination, plants initiate the formation of new organs (roots and leaves) and continue doing so during the entire vegetative growth phase (Fig 5). The embryo-to-vegetative transition is marked by the switching off and switching on of embryonic and cell cycle genes, respectively. RBR1 participates in both processes but in different ways. Through its interaction with PRC2 complexes, RBR1 facilitates the long-term maintenance of embryonic genes in a repressed state that depends on establishing a permanent chromatin landscape containing H3K27me3 (Gutzat *et al*, 2011) and therefore acts as a positive regulator of the embryo-to-seedling transition.

Activation of cell cycle gene expression occurs through an increase in CDK/cyclin activity leading to phosphorylation-dependent inactivation of RBR1's repressor function. This transition has been well studied during maize germination, where changes in the levels of a complex set of D-type cyclins and KRPs are crucial to control CDK activity (Garza-Aguilar *et al*, 2017; Godínez-Palma *et al*, 2017). D-type cyclins directly affect RBR1 phosphorylation levels and the release of E2F activity on target genes required to establish active cell proliferation and to initiate vegetative growth (Sánchez-Camargo *et al*, 2020).

The root apex consists of a distal zone in which cells are proliferating, the root apical meristem, and another more proximal area called transition zone, where cells switch to endocycles. E2FA in complex with RBR1 participates in the maintenance of a repressed state of genes such as *CCS52A1* and *CCS52A2* whose products are needed for the endocycle (Magyar *et al*, 2012). An analogous situation is found in leaves, where proliferation and endocycle stages are however temporally regulated: In young leaf primordia, most cells are dividing and subsequently enter the endocycle program to increase nuclear ploidy and to expand their size. Nutrient availability is crucial for organ growth in plants. The EbrB-3 BINDING PROTEIN (EBP1), which is partially regulated by the TOR pathway, promotes root growth in conditions of limiting sucrose supply and counteracts the RBR1-mediated switch to differentiation (Lokdarshi *et al*, 2020). Similar to its human counterpart, EBP1 interactors are RNA-binding proteins that participate in protein biosynthesis, another indication of the role of RBR1 in connecting cell growth and organogenesis in response to nutrient availability. Other pathways connecting RBR1 function to cell growth control, e.g., phosphorylation of RBR1 by casein kinase (Wang *et al*, 2016), are less well understood at the molecular level.

The role of activator E2F members in the transition from cell proliferation to the endoreplication phase (Fig 5) is balanced by the action of a repressor complex formed by E2FC together with RBR1 (del Pozo *et al*, 2002). E2FC is degraded by the proteasome, and overexpression of a stable form of E2FC negatively affects cell division by reducing the level of the DNA replication initiation protein CDC6 (Castellano *et al*, 2001), among other target genes (del Pozo *et al*, 2002). Conversely, reduction of E2FC leads to the formation of organs with more but smaller cells and a reduction in the nuclear ploidy level (del Pozo *et al*, 2006). Seedling growth after germination in the absence of light (skotomorphogenesis) is favored by high levels of E2FC and its DPB partner which restricts cell division and promotes endoreplication (del Pozo *et al*, 2002, 2006; López-Juez *et al*, 2007). Upon light availability (photomorphogenesis), the levels of both E2FC and DPB are drastically reduced by proteasome-dependent degradation mediated by the SCF^{SKP2a} ubiquitin ligase complex, thus allowing to restore active cell proliferation required for organ growth. Trichomes, the specialized epidermal cells, undergo several rounds of endoreplication associated with their genetically defined differentiation program. Among other transcription factors, this depends on the proper expression of *GLABRA1* (*GL1*), a gene than contains E2F binding sites in its promoter and therefore requires RBR1 activity. In this case, a feedback loop is likely active in the protodermal cells that differentiate into trichomes, since genes like RBR1 and SIAMESE (SIM) are direct targets of GL1 and GLABRA3 (GL3; Wenger & Marks, 2008; Morohashi & Grotewold, 2009).

Highly regulated levels of RBR1 are required for proper development of the shoot apical meristem (SAM) and for the growth of leaf primordia. Thus, local and transient overexpression of RBR1 in the SAM causes meristem cells to arrest cell division and initiate differentiation (Wyrzykowska *et al*, 2006). Later in leaf development, lack of RBR1 function in young leaves primarily engaged in cell proliferation leads to the production of extra cells, while in older leaves where endocycles predominate, an increase in nuclear ploidy is achieved (Park *et al*, 2005; Desvoyes *et al*, 2006). In young leaves, RBR1-E2FB complexes are abundant and of repressive nature, preventing endoreplication and cell differentiation; such complexes disappear concomitantly with leaf development (Henriques *et al*, 2010, 2013; Őszi *et al*, 2020). The tissue layer organization of leaves as well as their various cell types are also affected by RBR1. A direct consequence of the RBR1 role in organization of leaf cell layers is its relevance for leaf physiology (Fig 5), as demonstrated by RBR1 control over the amount and distribution of air spaces in-between mesophyll cells (Dorca-Fornell *et al*, 2013; Lehmeier *et al*, 2017). The regulatory network of the RBR1-E2F module is also important in other locations, e.g., for differentiation of the vascular system where RBR1 seems to play cell type-specific roles. Reduction of RBR1 produces a decrease in the number and increase in the size of cortical cells, while the number of xylem parenchyma cells increases (Jordan *et al*, 2007). This may be due to specific features in the differentiation timing of each cell type. Differentiation of xylem tracheary elements is blocked by XND1, a process that involves XND1 interaction with RBR1 via its LxCxD motif (Zhao *et al*, 2017a). It is possible that a complex RBR1-dependent loop operates in tracheary elements, since XND1 is a target of VND7 (Zhong *et al*, 2010), which in turn is regulated by the repressor E2FC (Taylor-Teeples *et al*, 2015). Therefore, it seems that the role of RBR1 in maintaining the balance between cell proliferation and differentiation during xylem development is mediated by XND1, VND7, and possibly other transcription factors, through their direct or indirect interaction with RBR1.

The transition from the vegetative shoot apical meristem to the floral meristem is another crucial phase during plant development (Fig 5). Through its interaction with PRC2 complexes, RBR1 regulates this growth phase transition (Johnston *et al*, 2008; Jullien *et al*, 2008; Borghi *et al*, 2010; Dumbliauskas *et al*, 2011). In monocotyledonous plants, their multiple RBR members have split roles in flowering. Rice RBR1 is necessary for the establishment and maintenance of the floral meristem and the inner floral organs, by controlling the expression of 10–12 floral homeotic genes, while RBR2 participates in stamen and pollen development (Duan *et al*, 2019). There are several other processes that are very important for the plant's life. Although less well studied, the transition from dormancy to growth in buds depends on the phosphorylation state of RBR1, with levels of phosphorylated RBR1 being high in dormant buds (Shimizu-Sato *et al*, 2008).

RBR1 and pathogen infection

As stated earlier, links between plant viruses (in particular geminiviruses and nanoviruses) and the RBR1 pathways date back to the discovery of RBR1 in plants, when mastreviruses (a geminivirus genus) were found to encode RepA, an RBR1-interacting protein utilizing a canonical LxCxE motif (Xie *et al*, 1995; Grafi *et al*, 1996; Horváth *et al*, 1998). Later, Rep proteins in begomoviruses (another geminivirus genus) were reported to lack an LxCxE motif but to still interact with RBR1 via a different amino acid motif (Ach *et al*, 1997a; Gutierrez, 2000; Arguello-Astorga *et al*, 2004). A protein binding RBR1 through a canonical motif, Clink, is also found in nanoviruses, a family of plant ssDNA viruses with a multipartite genome (Katul *et al*, 1998; Aronson *et al*, 2000). Interactions between geminivirus proteins and the RBR1 pathway are complex and not yet fully understood, since additional viral proteins like

AL3/REn (Settlage *et al*, 2001; Ruhel & Chakraborty, 2019) and C4 (Park *et al*, 2010; Zeng *et al*, 2018) also modulate the RBR1 pathway. Moreover, infectivity of geminiviruses requires further interactions with the host cell (McGivern *et al*, 2005; Arguello-Astorga *et al*, 2007).

Irrespective of the whether infections impinge only on the RBR1 pathway or also other pathways, profound changes in the host cells are a primary outcome (Hanley-Bowdoin *et al*, 2004; Ascencio-Ibanez *et al*, 2008). Modulation of the RBR1 pathway is also seen with various other types of pathogens (Depuydt *et al*, 2009; Stes *et al*, 2011; Wen *et al*, 2012; Villajuana-Bonequi *et al*, 2019). An unexpected link between the effector-triggering immunity (ETI), and its associated programmed cell death, and the RBR1-ERF module has been reported. The nuclear envelope protein CPR5 negatively regulates ETI by interacting with and suppressing CDK inhibitors, thereby increasing RBR1 hyperphosphorylation and, consequently, the release of E2F activity (Wang *et al*, 2014). However, further research is needed to better understand the full functional implications of this interaction.

RBR in regeneration and hyperplasia

Regeneration involves a general reprogramming of cells around the wounded area, to first resume cell proliferation and then initiate a process of cell fate acquisition to reform the new organ. It has been shown that the tissue damage response mediated by jasmonic acid is important for stem cell reactivation at least at two complementary levels, one is by impinging on the RBR/SCR pathway and another through activation of CYCD6;1 by the ERF109 transcription factor (Zhou *et al*, 2019). In fact, members of the ERF/AP2 family of transcription factors appear as key players in the regeneration process as shown for WIND1 (Iwase *et al*, 2011) and ERF115 (Heyman *et al*, 2016). Changes in transcriptional activity in the early response to wounding correlate with increased histone acetylation marks (H3K9ac and H3K14ac) deposited by members of the HAG1 family of acetyl transferases in various ERF family genes, including WIND1 (Rymen *et al*, 2019). Triggering of nuclear reprogramming during regeneration is counterbalanced by mechanisms that prevent unwanted cell dedifferentiation, which in a PRC2-dependent manner maintain WIND family members like WIND3 and embryonic genes like LEC2 in a repressed state (Ikeuchi *et al*, 2015). Given the tight link of RBR1 with histone deacetylases and PRC2, it is conceivable that RBR1 also plays a role in regulating regeneration capacity across various plant organs, as demonstrated for the RBR1-SCR module in the root stem cell niche (Zhou *et al*, 2019). Thus, the general strategy, although with different molecular players, is similar to the organ regeneration processes described in animals, e.g., in planarians, at the core of whose significant regenerative capacity lies the RB1-E2F module interacting with chromatin remodeling components (Zhu & Pearson, 2013). This does not mean that the RBR1-E2F module is the only pathway involved in reprogramming. Others are relevant during dedifferentiation and formation of giant cells in response to root-knot nematodes through genes controlling lateral root initiation (Olmo *et al*, 2020) or in plants of different ages through various hormonal pathways (Ye *et al*, 2020). These pathways frequently serve to regulate the expression of cell cycle genes at the transcriptional level (del Pozo *et al*, 2005).

A major difference to animals is that high regeneration potential is rather widespread in plants. In addition, their regeneration plasticity is enormous, since they can produce not only particular tissues, but entire organs or entire organisms from a single regeneration event. In all cases, the common path includes dedifferentiation, reprogramming, and cell proliferation and differentiation processes that are extremely well-coordinated in time and space.

But how do plants deal with extra cells produced during regeneration processes or as a consequence of misregulation of cell proliferation? Plants exhibit a high tolerance to changes in the level and function of key cell cycle genes, including those involved in DNA replication and chromatin dynamics, while such alterations frequently lead to embryonic lethality in animals. Although viruses, bacteria, and fungi can induce tumors in plants (Doonan & Sablowski, 2010), they do not lead to neoplastic transformation as we know it in animals. In fact, loss of the RB1 pathway function is a hallmark of many human cancer cells. In contrast, local loss of RBR1 leads to relatively mild phenotypes in vegetative organs of the adult, e.g., roots and leaves, where visible hyperplasia but no cell transformation occurs (Park *et al*, 2005; Wildwater *et al*, 2005; Desvoyes *et al*, 2006). In these cases, the most frequent behavior of excess cells is to interpret local positional information, acquire the cell fate of surrounding cells, and/or adjust the cell proliferation/endoreplication balance. This eventually allows growth into organs of near-normal size, containing more but smaller cells. At the same time, this plasticity of plants has to coexist with plant-specific features, such as the post-embryonic nature of their organogenesis, and their ability to form organs in a continuous manner throughout a plants' life. This could be potentially very dangerous when considering risks associated with perturbed cell proliferation control, since it involves numerous dedifferentiation/proliferation/differentiation decisions. In any case, it should be kept in mind that in plants, additional key factors likely contribute to the absence of neoplastic transformation as we understand it in animal cells. These include primarily the specific developmental mode of plants and the absence of metastasis, since cell movements inside the plant body are precluded by the presence of cell walls that maintain plant cells glued together and fixed at the very position where they were originally formed.

Future perspectives

Accumulating evidence gathered over the past 25 years demonstrates that RBR1 participates in multiple pathways, with a variety of roles at different levels of plant physiology. In all cases, its interactions with cellular factors, including transcriptional regulators, chromatin remodeling complexes or other interacting proteins, are at the molecular basis of RBR1 function. However, we still miss detailed insights into the fine regulation of RBR1 interactions, which frequently depend on RBR1 phosphorylation state modulated by various kinases. Therefore, identifying the role of RBR1 phosphorylation at specific sites as well as the CDK/cyclin complexes involved would be necessary to gain more information about how and when during the cell cycle or during differentiation phospho-RBR1 isoforms exert their action. Such information shall also help to understand the kinetics of complex formation with the different RBR1-interacting factors. Given the conservation of amino acid

sequences around potential phosphorylation sites, a possible way to explore is the use of antibodies available for phosphorylated forms of human RB1 in combination with the generation of site-specific phosphorylation mutants. These and other protein interactions of RBR1 depend on the presence of intrinsically disordered RBR1 regions, which could be crucial in the formation of protein condensates by liquid-liquid phase separation.

The understanding of the role of RBR1 complexes with transcription factors of different families is still at its beginning. Following identification of a given interaction, the dynamics of such interaction should provide information about spatial and temporal activity of a given such complex and help to explain the role of RBR1 in different organs and at different developmental stages. Such studies need to be combined with detailed knowledge of RBR1 complex availability, e.g., its protein expression, modification, and degradation. Thus, identification of E2F targets and the various E2F family members in relation to RBR1 will need to be done not only genome-wide, but also at an individual scale, in order to reveal the basis for their spatial and temporal regulation, which is highly dependent on the target under study. In this context, the role of DREAM complexes and their targets in controlling cell cycle progression and the transition to differentiated states need to be understood at the molecular level. Again, a detailed map of RBR1 interactions and the individual circuitries at the cellular level is still far from being understood. Furthermore, expanding these studies to the response of plants to their environment should be important for the understanding of RBR1 roles at the organismal and population levels.

In summary, there are numerous avenues to be explored at the molecular, cellular, developmental, and environmental levels before we obtain a general picture of the multifunctional roles played by RBR1 in plant physiology. The future should see rich advances in these and other directions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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