



# RETINOBLASTOMA-RELATED interactions with key factors of the RNA-directed DNA methylation (RdDM) pathway and its influence on root development

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## Abstract

**Main Conclusion** Our study presents evidence for a novel mechanism for RBR function in transcriptional gene silencing by interacting with key players of the RdDM pathway in Arabidopsis and several plant clades.

**Abstract** Transposable elements and other repetitive elements are silenced by the RNA-directed DNA methylation pathway (RdDM). In RdDM, POLIV-derived transcripts are converted into double-stranded RNA (dsRNA) by the activity of RDR2 and subsequently processed into 24 nucleotide short interfering RNAs (24-nt siRNAs) by DCL3. 24-nt siRNAs serve as guides to direct AGO4–siRNA complexes to chromatin-bound POLV-derived transcripts generated from the template/target DNA. The interaction between POLV, AGO4, DMS3, DRD1, RDM1 and DRM2 promotes DRM2-mediated de novo DNA methylation. The Arabidopsis Retinoblastoma protein homolog (RBR) is a master regulator of the cell cycle, stem cell maintenance, and development. We in silico predicted and explored experimentally the protein–protein interactions (PPIs) between RBR and members of the RdDM pathway. We found that the largest subunits of POLIV and POLV (NRPD1 and NRPE1), the shared second largest subunit of POLIV and POLV (NRPD/E2), RDR1, RDR2, DCL3, DRM2, and SUVR2 contain canonical and non-canonical RBR binding motifs and several of them are conserved since algae and bryophytes. We validated experimentally PPIs between Arabidopsis RBR and several of the RdDM pathway proteins. Moreover, seedlings from loss-of-function mutants in RdDM and RBR show similar phenotypes in the root apical meristem. We show that RdDM and SUVR2 targets are up-regulated in the 35S:AmiGO–RBR background.

**Keywords** Epigenetics · de novo DNA methylation · Gene silencing · RBR · Root development · Viridiplantae

## Abbreviations

AGO4 Argonaute 4  
DCL3 Dicer-like 3

DRM2 De novo DNA methyltransferase domains rearranged 2  
LTR Long Terminal Repeat  
NRPD Nuclear RNA Polymerase D  
POLIV Polymerase IV  
POLV Polymerase V  
PRC2 Polycomb repressor complex 2

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RBR	Retinoblastoma-related
RdDM	RNA-directed DNA methylation pathway
RDM1	RNA-directed DNA methylation 1
RDR2	RNA-dependent RNA polymerase 2
SLiM	Short linear motif
Y2H	Yeast two-hybrid

## Introduction

DNA methylation is essential for proper development in eukaryotes. In plants, it is involved in the regulation of gene expression, and the defense against invasive nucleic acids, both with effects on development and physiology. In plants, cytosines can be methylated in symmetrical (CG or CHG) and asymmetrical (CHH) sequence contexts (where H can be A, T, or C). Transposable elements (TEs) and other repetitive sequences are the main targets of DNA methylation (Matzke and Moshier 2014; Borges and Martienssen 2015). The major small RNA-mediated epigenetic pathway involved in de novo DNA methylation is the RNA-directed DNA methylation (RdDM) pathway (Matzke and Moshier 2014; Erdmann and Picard 2020). RdDM involves the function of Nuclear RNA Polymerase D (NRPD) or POL IV and NRPE or POLV (Hagg and Pikaard 2011). POLIV transcribes short single-stranded RNA (ssRNA) 26 to 45 nt in length (from the target locus that will be methylated) that serves as the substrate for RNA-Dependent RNA Polymerase 2 (RDR2) for the generation of double-stranded RNA (dsRNA). The resulting dsRNA is processed by Dicer-Like 3 (DCL3) into 24-nt small interfering RNAs (siRNAs). HUA ENHANCER 1 (HEN1) methylates 24-nt siRNAs at their 3'-end and are subsequently recruited by ARGONAUTE 4 (AGO4) (or another close paralog such as AGO6 and AGO9). The AGO4–siRNA complex associates with chromatin-bound POLV-dependent transcripts produced from the same loci that will be methylated, through RNA–RNA pairing. The association between the AGO4–siRNA complex and POLV is further stabilized by protein–protein interactions (PPIs) between AGO4 and the CTD of POLV. Recruitment of the de novo DNA methyltransferase DOMAINS REARRANGED 2 (DRM2) to the template/target DNA occurs through the activity of RNA-DIRECTED DNA METHYLATION 1 (RDM1) that can bind methylated single-stranded DNA (ssDNA) and interacts with DRM2 and AGO4 (reviewed in Matzke and Moshier 2014; Trujillo et al. 2018).

Retinoblastoma proteins are multi-faceted master regulators of cell reprogramming in eukaryotes and are involved in the control of cell cycle, DNA damage response, and in protein–protein interactions (PPIs) with transcription factors that modulate stem cell maintenance and asymmetric cell division for proper cell lineage commitment (Calo et al. 2010; Cruz-Ramírez et al. 2012; Harashima and Sugimoto

2016; reviewed in Dyson 2019; reviewed in Desvoyes and Gutiérrez 2020). In Arabidopsis, RETINOBLASTOMA RELATED (RBR) has been shown to bind DNA, putatively regulates the transcription of hundreds of genes and transposable elements (Bouyer et al. 2018), and also indirectly modulates gene expression by PPIs and genetic interactions with lineage-specific transcription factors (Cruz-Ramírez et al. 2012, 2013; Matos et al. 2014; Zhao et al. 2017a, b), chromatin-remodeling factors such as PICKLE (PKL) (Ötvös et al. 2021), and the POLYCOMB REPRESSOR COMPLEX 2 (PRC2) (Julien et al. 2018). The PRC2 complex regulates plant growth and development through the trimethylation of lysine 27 on histone 3 (H3K27me3), a well-known epigenetic mark involved in transcriptional repression. Two independent studies have established the connection between RBR and PRC2. Jullien et al. (2008) demonstrated that RBR directly binds to MULTICOPY SUPPRESSOR OF IRA1 (MSI1), an essential component of Arabidopsis PRC2 protein complexes involved in female gametogenesis, seed, and vegetative development. The RBR–MSI1 complex directly represses *DNA METHYLTRANSFERASE 1 (MET1)* transcription. MET1 is a DNA methyltransferase acting on cytosine methylation at symmetrical CpG positions. MET1 repression occurs only on the female gamete and is required for the expression of imprinted genes. A similar observation was also reported by Johnston et al. (2008). The interaction between RBR and PRC2 is potentially deeper since FERTILIZATION-INDEPENDENT ENDOSPERM (FIE). Another member of the PRC2 complex that interacts with MEDEA (MEA), SWINGER (SWN), and CURLY LEAF (CLF) (Oliva et al. 2016) does contain a highly conserved LxCxE motif, which is characteristic of proteins that bind with high-affinity to RBR (Cruz-Ramírez et al. 2012).

Plant and animal Retinoblastoma proteins share conserved residues that allow them to interact with proteins containing a LxCxE SLiM (SLiM: Short Linear Motif) RBR-binding motif (Lee et al. 1998; Dick 2007). A decade ago, a global search in the Arabidopsis proteome for proteins containing the LxCxE SLiM led us to the identification of hundreds of candidates that potentially interact with the single Arabidopsis Retinoblastoma protein: RBR. By employing the LxCxE motif, which confers high affinity to RBR, as an in silico bait to identify Arabidopsis RBR protein partners (Cruz-Ramírez et al. 2012), we identified several components of the RdDM pathway including the largest subunits of POLIV and POLV, RDR1, RDR2, DCL3, DRM2 and SUV2 as potential targets of RBR. In this study, we demonstrate that RBR binds to DRM2, DRD1, and SUV2. We also report that seedlings of loss-of-function mutants in RBR and genes of the RdDM pathway exhibit phenotypes in the root apical meristem including defects in the root stem cell niche (RSCN). This is consistent with the observation

that RdDM and SUV2 targets are up-regulated when RBR is post-transcriptionally silenced using the cell-type-specific artificial microRNA for Gene-silencing Overcome (amiGO) system (Cruz-Ramírez et al. 2013). Our results uncover a novel mechanism for RBR function in transcriptional silencing through its interactions with key components of the RdDM pathway and open the possibility of a convergent action of RBR–DRM2 in the regulation of TEs and lineage or tissue-specific transcription factors, and stem cell regulators, such as *WUSCHEL*, *AGAMOUS LIKE 15* (*AGL15*), and *POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION* (*POLAR*), among other interesting putative target genes.

## Materials and methods

### Plant materials

To analyze root phenotypes and expression patterns, *Arabidopsis thaliana* plants were grown as described in Cruz-Ramírez et al. (2004). Col-0 wild type, double (*nprpd2a-2;nprpd2b-1*) and triple mutants (*drm1;drm2;cmt3*) plants were used for phenotypic analyses, as well as transgenic lines (*pRBR::RBR:CFP*, *pDRM2::DRM2-GFP* and *35S::AmiGORBR*) (Cruz-Ramírez et al. 2012, 2013; Jullien et al. 2012).

### Meristem size analysis

To define the effect of diverse mutations on root phenotype, the root meristem size was determined by counting the number of cortical cells from the quiescent center to the first elongated cortex cell, as described in Dello et al. (2007).

### Microscopic analysis

To determine the spatio-temporal expression patterns of diverse transgenic lines, seedlings were grown, and roots were prepared for confocal microscopy as previously described (Cruz-Ramírez et al. 2012). Fluorescent signals for the diverse genetic backgrounds were recorded with a Leica SP2 CLSM and a Zeiss LSM 800 CLSM. To determine root phenotypes in the root apical meristem (RAM) of wild type and mutant lines, roots were mounted and stained with Lugol as in Willemsen et al. (1998) and were visualized by Nomarski optics.

### Protein–protein interaction (PPI) assays

To validate that a subset of the LxCxE-containing proteins are true direct RBR interactors we performed Yeast two-hybrid (Y2H) assays by employing the ProQuest Two-Hybrid System

(Invitrogen Life Technologies) as reported in Cruz-Ramírez et al. (2013). To quantify the strength of each interaction, three biological and technical replicates of beta-galactosidase assays with CPRG as substrate were performed. With the same aim, Bimolecular Fluorescence Complementation Assays in Arabidopsis protoplast were performed as reported in Cruz-Ramírez et al. (2012). For RBR–DRM2, RBR–DRD1 and controls YFP fluorescence was recorded with a Leica SP2 CLSM.

### Computational analyses and ortholog identification

To explore the conservation along viridiplantae of LxCxE-containing proteins that participate in RdDM, Angiosperm protein sequences were downloaded from Phytozome (<https://phytozome-next.jgi.doe.gov/>), while non-angiosperm and algae protein sequences were downloaded from Phytozome, Fernbase (Li et al. 2018), TreeGenes (Wegrzyn et al. 2019), and Phycocosm (Grigoriev et al. 2021). Sequences for *A. agrestis* and *P. margaritaceum* were downloaded directly from the University of Zurich Hornworts database (Li et al. 2020: <https://www.hornworts.uzh.ch/en.html>) and the Penium genome database (Jiao et al. 2020: <http://bioinfo.bti.cornell.edu/cgi-bin/Penium/blast.cgi>), respectively. LxCxE–SLiM containing protein sequences were detected using a custom PERL script. To infer orthologues, all protein sequences from all 28 species analyzed were placed into orthogroups using the OrthoFinder software (Emms et al. 2019).

### qRT-PCR assays of RdDM targets

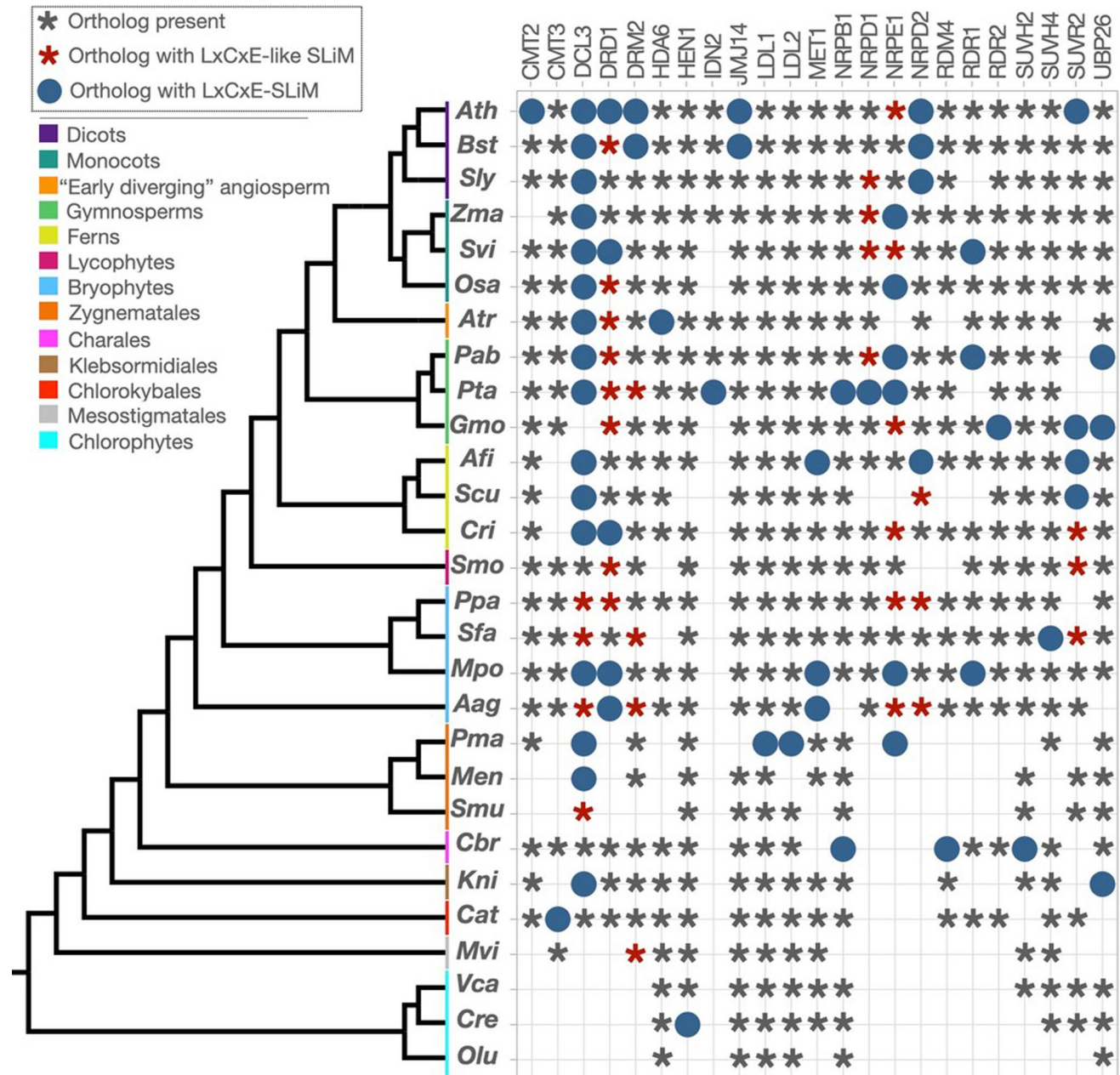
With the aim of determining if *RBR* downregulation can affect the transcription of known RdDM and DRM2 reported targets, twenty seedlings of 14-day-old post-germination plants from Col-0 or *35S::AmiGORBR* (Cruz-Ramírez et al. 2013) were used for total RNA extraction by *TRIzol* reagent (ThermoFisher) in three biological replicates. Five micrograms of total RNA per 20 µL reaction was used to generate cDNAs according to the manufacturer's protocol for SuperScript II (ThermoFisher). The expression level was determined using SYBR GREEN mix (ThermoFisher) in a 10 µL reaction. The data were normalized using Actin 7 expression levels. The primers used in these experiments are those reported in Han et al. (2014) and Supplementary Table S1. The expression levels for every transcript were obtained from biological and technical triplicates, and the data plotted represents the average of these triplicates to which standard deviation was applied.

## Results

### Major players of the RdDM pathway and their putative RBR-binding motifs

Early predictions for Arabidopsis RBR-interactors served as the basis for the functional characterization of the interaction between RBR with diverse lineage-specific factors such as SCARECROW, FAMA, XND1, and PICKLE,

among others (Cruz-Ramírez et al. 2012; Matos et al. 2014; Zhao et al. 2017a, b; Zhou et al. 2019; Ötvös et al. 2021). In addition to the aforementioned proteins, we identified many proteins with diverse key molecular and cellular functions bearing the RBR-binding motif which, in many cases, were evolutionarily conserved. Among them, we found that components of the RdDM pathway including NRPD2, DRD1, DRM2, DCL3, and SUVR2 contain the canonical LxCxE SLiM (Fig. 1, Suppl. Table S2). We also found that major players of the RdDM pathway including



**Fig. 1** Phylogenetic conservation of canonical (solid blue circle) and non-canonical (red asterisk) LxCxE SLiMs in orthologs of the RdDM pathway in representative species along Viridiplantae



NRPD1, NRPE, and RDR2 contain a non-canonical RBR-interaction motif I/LxFxE (Fig. 1, Suppl. Table S2). The observation that eight components of the RdDM pathway shared canonical and non-canonical RBR-interaction motifs prompted us to investigate if some of them are true physical RBR interactors.

### Conservation of LxCxE-like motifs in RdDM factors along Viridiplantae

To gain insight into the evolutionary conservation of the LxCxE SLiM present in components of the RdDM pathway, we interrogated publicly available plant and algae genomes aiming to detect the presence of canonical and non-canonical (IxCxE/LxCxD/IxCxD) LxCxE SLiMs among orthologs of the RdDM pathway along the Viridiplantae clade. To optimize the breadth of the plant phylogeny to cover, we focused on a small subset of species with available sequenced genomes representing each major lineage of the Viridiplantae. The species selected and analyzed include representatives from angiosperms (*A. thaliana* [Ath]; *Boechera stricta* [Bst]; *Solanum lycopersicum* [Sly]; *Zea mays* [Zma]; *Setaria viridis* [Svi]; *Oryza sativa* [Osa]; *Amborella trichopoda* [Atr]), gymnosperms (*Picea abies* [Pab]; *Pinus taeda* [Pta]; *Gnetum montanum* [Gma]), ferns (*Azolla filliculoides* [Afi]; *Salvinia cucullata* [Scu]; *Ceratopteris richardii* [Cri]), lycophytes (*Selaginella moellendorffii* [Smo]), bryophytes (*Sphagnum fallax* [Sfa]; *Physcomitrium patens* [Ppa]; *Marchantia polymorpha* [Mpo]; *Anthoceros agrestis* [Aag]) charophyte (*Penium margaritaceum* [Pma]; *Mesotaenium endlicheranium* [Men]; *Spirogloea muscicola* [Smu]; *Chara braunii* [Cbr]; *Klebsormidium nitens* [Kni]; *Chlorokybus atmophyticus* [Cat]; *Mesostigma viride* [Mvi]) and chlorophyte (*Volvox carteri* [Vca]; *Chlamydomonas reinhardtii* [Cre]; *Ostreococcus lucimarinus* [Olu]) algae.

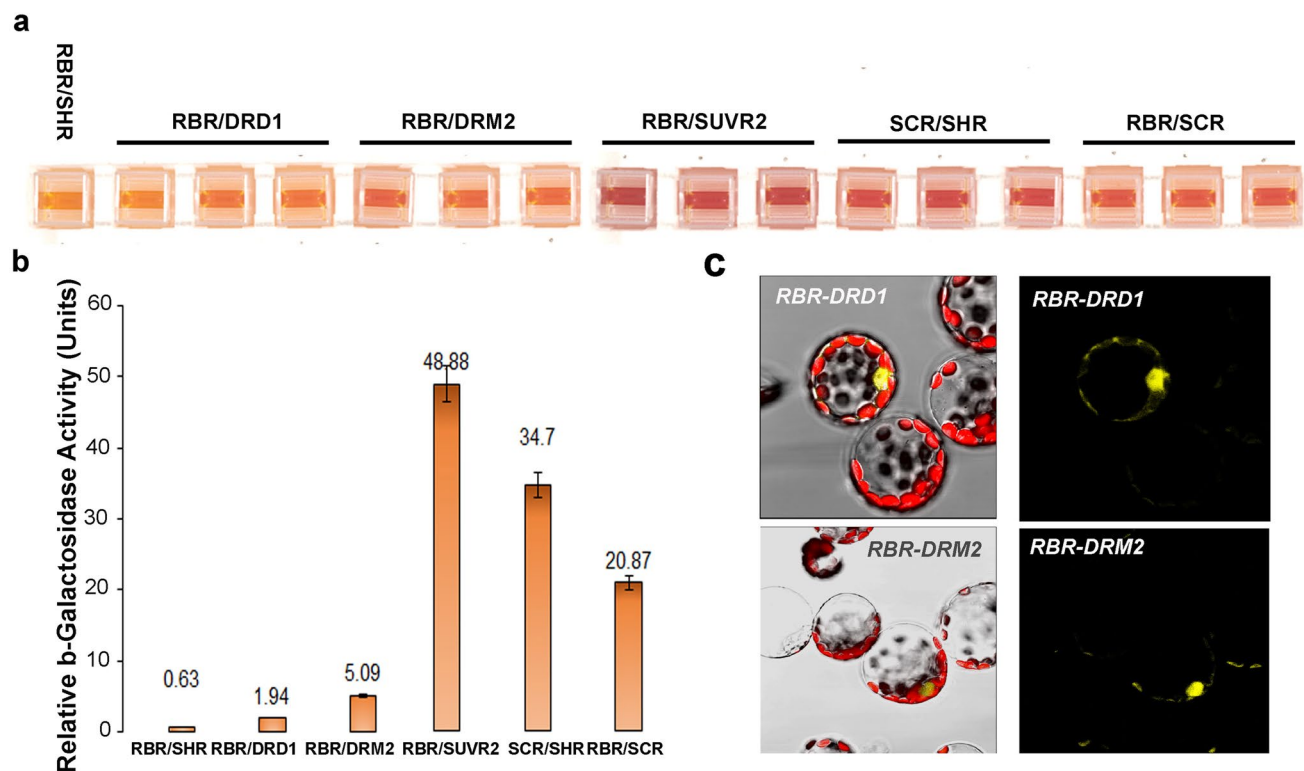
Our analysis revealed that *A. thaliana* was the species with more proteins containing either canonical or non-canonical RBR-binding motifs (Fig. 1, Suppl. Table S2), with 8 out of 23 RdDM-related proteins analyzed (DCL3, DRD1, DRM2, NRPD2, SUVR2, CMT2, JM14 and NRPE1). DCL3 orthologs showed the highest level of conservation for canonical and non-canonical LxCxE SLiMs among the species analyzed as they are absolutely conserved in tracheophytes, with the only exception of *G. montanum*. Interestingly, while DCL3 in *M. polymorpha* bears a canonical LxCxE SLiM, DCL3 orthologs in other bryophytes specifically *S. fallax*, *P. patens*, and *A. agrestis* contain LxCxE-like SLiMs. Of the seven charophyte algae species analyzed, 3 of them contain canonical RBR-binding motifs (*P. margaritaceum*, *M. endlicheranium*, *K. nitens*), while *S. muscicola* contains a LxCxE SLiM (Fig. 1, Suppl. Table S2). Although the LxCxE SLiM is highly conserved along DCL3

orthologs, it is difficult to determine if the canonical or the non-canonical motif is the ancestral one.

DRD1 orthologues showed the presence of the LxCxE SLiM in a patchy pattern along the plant lineages analyzed. The presence of the LxCxE SLiM in DRD1 orthologues is less conserved than in DCL3 orthologues, since we were not able to find LxCxE or LxCxE-like SLiMs in any of the algae species analyzed; however, it is present in *Marchantia*, *Anthoceros*, and *Ceratopteris* DRD1 orthologs (Fig. 1, Suppl. Table S1). The presence of the LxCxE SLiM is even less conserved in DRM2 orthologs than in DRD1, with only two DRM2 orthologs from *Arabidopsis* and *Boechera* exhibiting a canonical SLiM and non-canonical LxCxE SLiMs present in *Pinus*, *Sphagnum*, *Anthoceros* and *Mesostigma*. In the case of the subunits of POLIV and POLV, we expanded a presence–absence analysis along the plant phylogeny, similar to that reported previously by Huang et al. (2015). We found that NRPE1, NRPD1 and NRPD2 showed the presence of both canonical and non-canonical LxCxE SLiMs in diverse species, among these 3 proteins we found that NRPE1 is the one with more species containing either canonical or non-canonical RBR-binding SLiM (Fig. 1, Suppl. Table S2). While *Arabidopsis* NRPD1 does not contain a LxCxE SLiM, *P. taeda* NRPD1 ortholog contains a canonical LxCxE SLiM and orthologs from *S. viridis*, *P. abies*, maize and tomato bear a non-canonical LxCxE SLiM. We observed the presence of canonical LxCxE SLiMs in NRPE1 from charophyte to flowering plants (*P. margaritaceum*, *M. polymorpha*, *P. abies*, *P. taeda*, *Z. mays* and *O. sativa*) and non-canonical LxCxE SLiMs in NRPE1 orthologs from *C. richardii*, *A. agrestis*, *P. patens*, *G. montanum*, *S. viridis* and *A. thaliana*. The presence of canonical and non-canonical LxCxE SLiMs involved in RBR-binding in the POLIV and POLV largest subunits (NRPD1 and NRPE1, respectively) and the shared second largest subunit (NRPD/E2) strongly suggests that a new layer of regulation of the RdDM pathway mediated by RBR is present in land plants.

### DRM2, DRD1 and SUVR2 physically interact with RBR

Based on their conservation patterns we selected a group of proteins to test for protein–protein interactions with RBR. We generated constructs using amplified coding sequences (CDS) of DRM2, DRD1 and SUVR2 from *Arabidopsis* for Y2H assays, to test if they interact with RBR (previously cloned in pDEST32 and used in Cruz-Ramírez et al. 2012). Our results showed that SUVR2 strongly interacts with RBR when quantified and compared with other partners and controls (Fig. 2a, b) but DRD1 and DRM2 showed weak interaction. The previously described Y2H results prompted us to confirm DRD1–RBR and DRM2–RBR interactions by Bimolecular Fluorescence Complementation (BiFC) assays.



**Fig. 2** **a, b** Yeast two-hybrid analyses showing beta-galactosidase colorimetric reaction **a** and its quantitation **b**, for diverse proteins of the RdDM pathway and RBR. SCR–RBR and SCR–SHR combina-

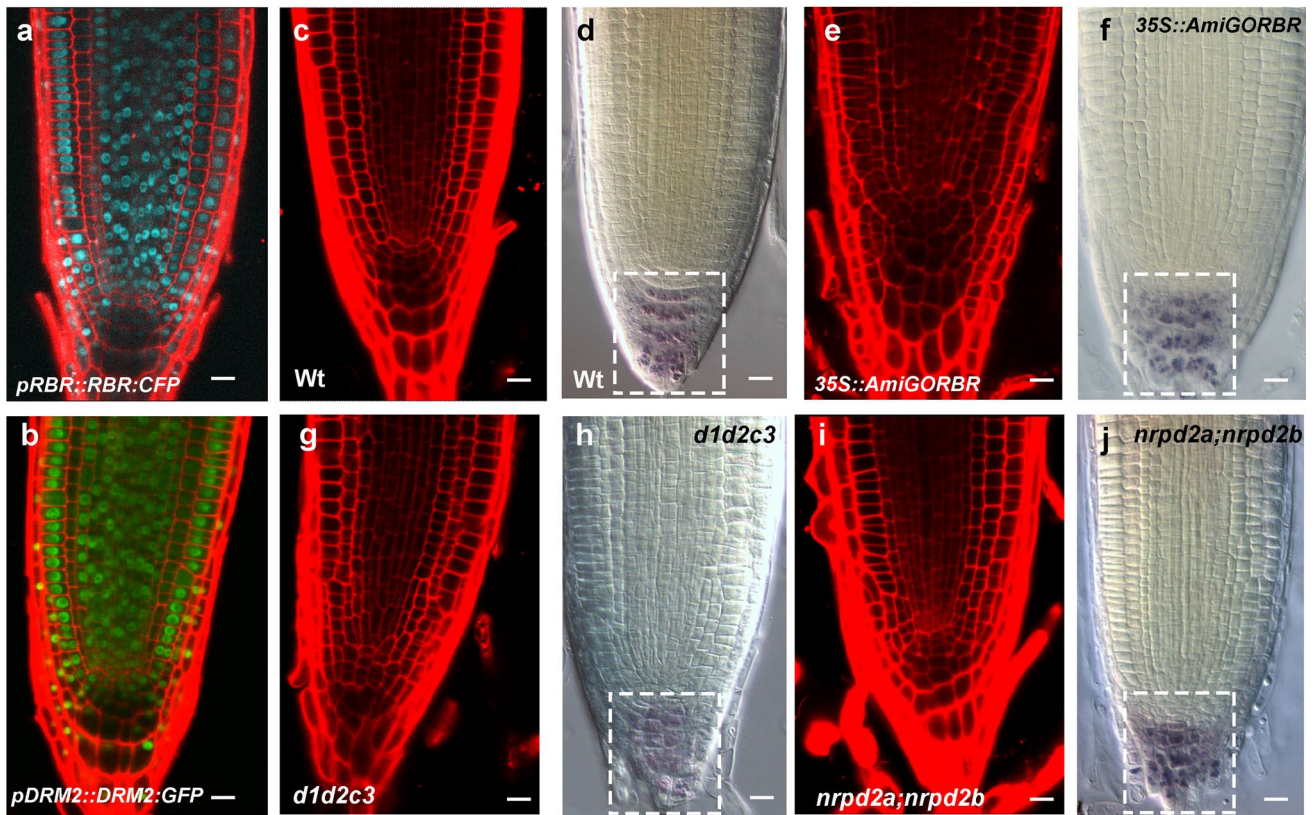
tions are positive controls, and RBR–SHR is the negative control (Cruz-Ramírez et al. 2013). **c** RBR–DRD1 and RBR–DRM2 binding by BiFC in *Arabidopsis* mesophyll protoplasts

We found that YFP nuclear signal is evident in *Arabidopsis* mesophyll protoplasts, confirming that DRD1 and DRM2 do interact with RBR. We also found that the *M. polymorpha* DCL3 ortholog interacts with both *Arabidopsis* and *M. polymorpha* RBRs by Y2H assays (León-Ruiz and Cruz-Ramírez 2022). Further experimental work is required to confirm PPIs between RBR and other RdDM-related proteins including NRPD1, NRPE1 and NRPD/E2, but it is important to consider that regulation by RBR can go beyond its direct interactors, as will be discussed later. Taken together, our results indicate that the evolutionary conservation of LxCxE SLiMs among components of the RdDM pathway is consistent with our experimentally validated interactions with RBR in the cases of DRD1, DRM2 and SUVR2.

### RdDM and RBR loss-of-function mutants show similar developmental alterations

In addition to their physical interaction, RBR and DRM2 protein fusions (pRBR::RBR:CFP, pDRM2::DRM2:GFP) have quite similar expression patterns as both proteins are present in every cell of the RAM (Fig. 3a, b). Since RBR has been shown to regulate stem cells and QC divisions in the *Arabidopsis* RAM (Cruz-Ramírez et al. 2012, 2013), we wondered if loss of function (LOF) mutants, in tested

and putative interactors, in genes of the RdDM pathways may display similar phenotypes to those in RBR LOF lines. Therefore, we analyzed root development of 12 dpv (days post-germination) seedlings of the *drm1;drm2;cmt3* triple mutant and the *nRPD2a;nRPD2b* double mutant and observed that primary root development in these mutants is affected. Although the phenotype is variable among seedlings from mild to severe, they all exhibit shorter roots (Fig. 4a–c) and a shorter meristematic zone (Fig. 4d–f). We analyzed in detail the organization of the RAM and root stem cell niche (RSCN) of *drm1;drm2;cmt3* and *nRPD2a;nRPD2b* 10 dpv seedlings and observed that roots from both mutant lines showed a disorganized RAM and defects in the columella region relative to wild-type seedlings (Fig. 4d, e). In the case of *nRPD2a;nRPD2b* such phenotypes correlate with aberrant expression patterns of WOX5 and DR5 markers, suggesting that although the meristematic activity is not fully lost, it is affecting the proper stem cell niche activity, compared to the WT (Figs. 4f–i, 3i, j). Such alterations correlate well with phenotypes observed in *drm1;drm2;cmt3* and *35S::AmiGO-RBR* roots such as QC divisions, extra stem cells and aberrant divisions and alterations in the columella region (Fig. 3e–h). Similar RBR loss-of-function phenotypes have been extensively described in by Wildwater et al. (2005) and Cruz-Ramírez et al. (2013).



**Fig. 3** Longitudinal root sections of 10 dpg seedlings imaged by confocal laser scanning microscope (CLSM) **a–i** and Nomarski optics of lugol-stained roots **d, f, h** and **j**. Panels **a** and **b** show the expression patterns of *pRBR::RBR:CFP* and *pDRM2::DRM2:GFP*. **c** confocal and **d** Nomarski optics images showing root apical meristem (RAM) and root stem cell niche (RSCN) organization in Col-0 (WT) seedlings. **e** Confocal and **f** Nomarski images of *35S::AmiGO-RBR* seedlings showing alterations in the RAM and RSCN. **g** Confocal and **h** Nomarski images of *drm1;drm2;cmt3 (d1d2c3)* triple mutant seedlings showing alterations in the RAM and the RSCN. **i** Confocal and **j** Nomarski images of *nrpd2a;nrpd2b* double mutant seedlings showing phenotypes in the RAM and RSCN, dotted squares highlight the Columella region. Bars in all panels = 50 μm

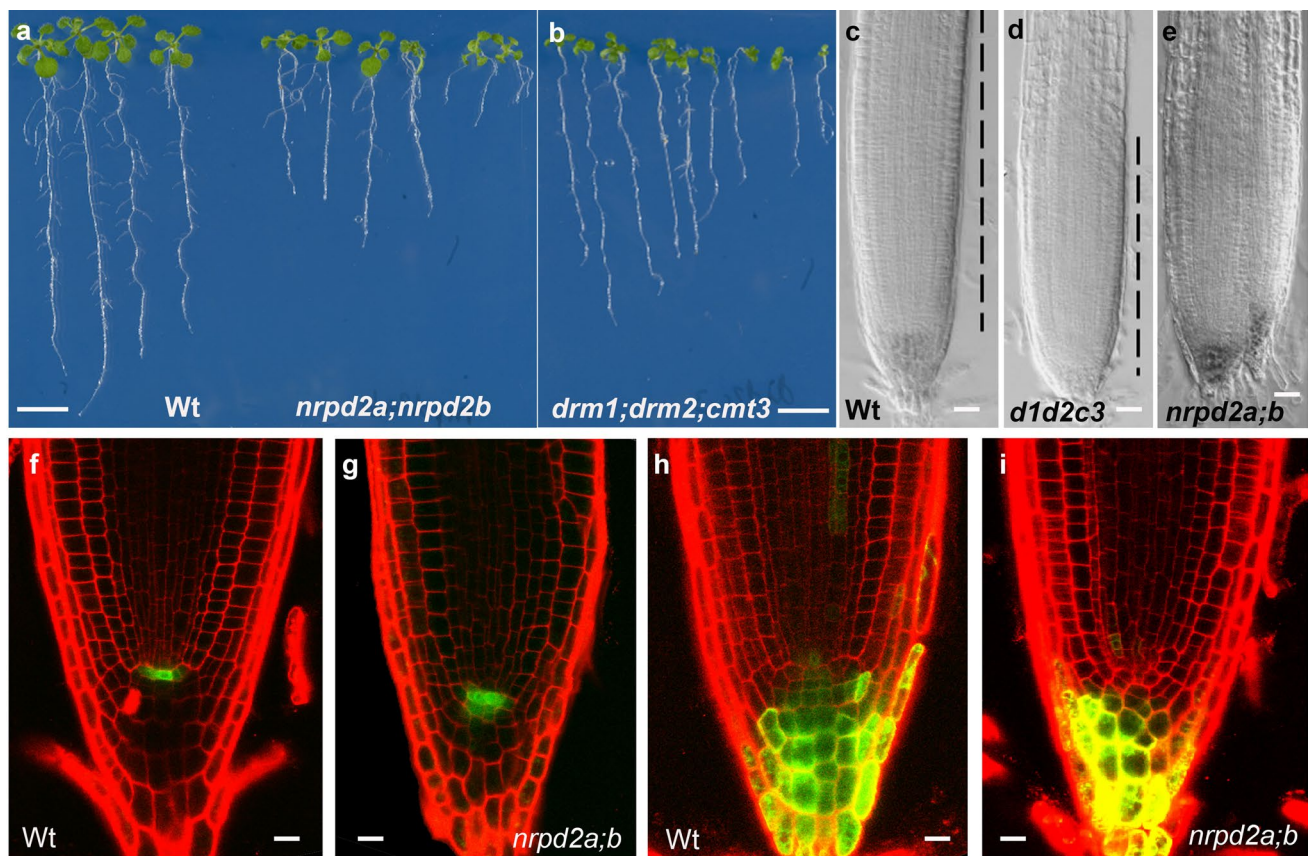
### RdDM and SUVR2 targets are up-regulated in the AmiGO-RBR background

SUVR2 silences a subset of RdDM target loci, as well as RdDM-independent targets (Han et al. 2014). Well-known targets of RdDM include TEs from the solo LTR (SLTR) and AtGP1 LTR families and genes such as *SUPPRESSOR OF drm1 drm2 cmt3 (SDC)*. It has been shown that at *SDC* and *ERT7* loci, the *suvr2* loss-of-function mutants display a synergistic phenotype with mutants in key genes of the RdDM pathway, which suggests that at these loci SUVR2 might exert silencing through a pathway which is partially independent of RdDM (Han et al. 2014). Our data indicates that SUVR2, DRM2 and DRD1 bind in vitro to RBR and based on the presence of the LxCxE SLiM other RdDM components like NRPD1, NRPE1 and DCL3 could also potentially bind to RBR. Therefore, we wondered if the downregulation of RBR may affect the expression of several known genes, which are targets of, and repressed by, RdDM and SUVR2. To answer such question, we isolated total RNA

of 12-day-old wild-type and *35S::AmiGO-RBR* seedlings and performed qRT-PCR assays using previously reported primers for *SDC*, *AtGP1*, *solo LTR (SLTR)*, AT1TE51360 (AT1TE), AT2TE78930 (AT2TE), *ERT7*, *ERT9*, *ERT12*, and *ERT14*. Our results showed that all tested loci are either moderately or strongly up-regulated in the RBR loss-of-function background relative to the wild-type control (Fig. 5a). It has been shown that *ERT9* transcripts are not de-repressed in the *suvr2* mutant background, suggesting that RBR might influence DRM2 and SUVR2 targets independently. Overall, these results indicate that RBR acts by repressing RdDM and SUVR2 transposable elements targets. Whether this action depends on RBR protein–protein interaction with DRM2, SUVR2 or DRD1 remains to be answered in future studies.

The RdDM pathway methylates not only TE loci, but also hundreds of protein-coding genes (Jha and Shankar 2014). Since RBR also has hundreds of targets, predicted by Chip-Seq (Bouyer et al. 2018), we explored a potential overlap between the 4,431 DRM2 methylation targets





**Fig. 4** Root and shoot phenotypes, recorded with stereomicroscope, of 10-day-post germination (dpg) seedlings of wild-type **a**, double and triple mutants in RdDM proteins **a**, **b**. Nomarski optics for RAM phenotypes of *drm1;drm2;cmt3* and *nrpd2a;nrpd2b* mutant **d**, **e** and wild-type **c** roots of 10 dpg seedlings. Longitudinal root sections

of 10 dpg seedlings by confocal laser scanning microscope (CLSM) of *pWOX5::GFP* and *TCS::GFP* transgenes in WT **f**, **h** and *nrpd2a;nrpd2b* **g**, **i** backgrounds, respectively. Bars = 1 cm **a**, **b**, 150  $\mu$ m **c**–**e**, 50  $\mu$ m **f**–**i**

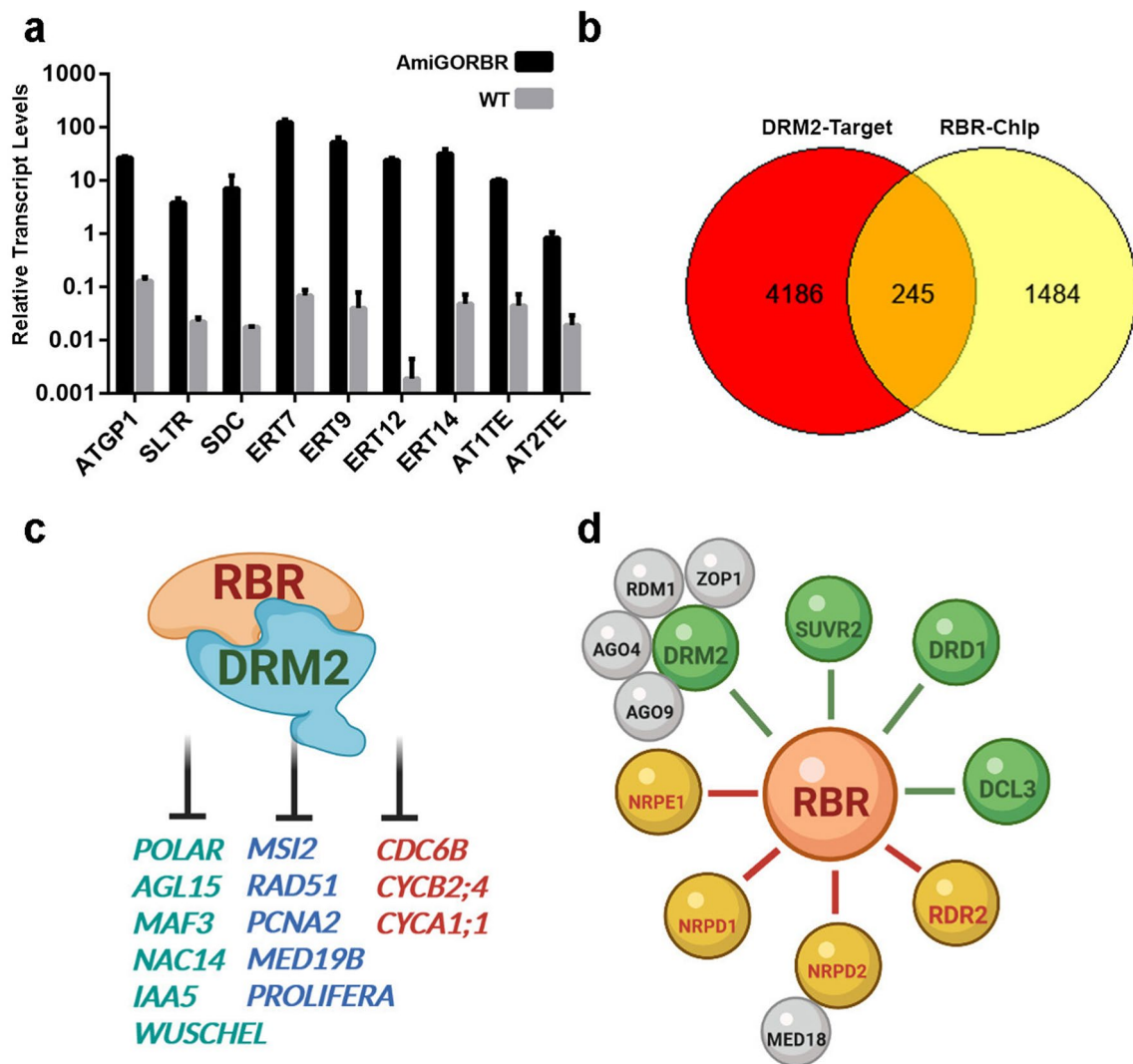
proposed by Jha and Shankar (2014) and the 1,729 RBR target genes predicted by Bouyer et al. (2018). We found that 245 target genes are shared between RBR and DRM2 (Fig. 5b). Among the 245 shared target genes we found several interesting ones (Fig. 5c). We highlighted those that encode transcriptional regulators such as *POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION (POLAR)*, *AGAMOUS LIKE 15*, *NAC15*, *INDOLE-3-ACETIC ACID INDUCIBLE 5 (IAA5)* and *MADS AFFECTING FLOWERING 3 (MAF3)*. Another important transcription factor that has been shown to act downstream RBR is *WUSCHEL*, the role of this putative interaction between RBR and WUS and with the RdDM pathway will be discussed later. We also found that genes related to DNA integrity, DNA replication and cell cycle are common targets of RBR and DRM2, such as *RAD51*, *PROLIFERATING CELL NUCLEAR ANTIGEN 2 (PCNA2)*, *MINICHROMOSOME MAINTENANCE 7/PROLIFERA (MCM7)*, *MS1*, *MEDIATOR 19B (MED19B)*, *CYCLIN A1;1 (CYCA1;1)*, *CELL DIVISION CONTROL 6B (CDC6B)* and

*CYCLIN B2;4 (CYCB2;4)* (Suppl. Table S3). The fact that several cell cycle-related genes are common targets of RBR and RdDM correlates with alterations observed in roots of LOF mutants in RBR and RdDM-genes.

## Discussion

This study uncovers novel mechanisms for RBR through the interaction with components of the RdDM pathway. We found that, in Arabidopsis, eight proteins involved in the RdDM pathway shared canonical and non-canonical RBR-interaction motifs and we demonstrate direct interactions between RBR and a subset of them. Moreover, besides the validated and the putative direct RBR interactors, RBR could affect other PPIs indirectly as documented in the IntAct Molecular Interactions Database from EMBL-EBI, since DRM2 establishes 12 PPIs, from which at least 4 are direct interactions with members of the RdDM pathway,





**Fig. 5** Transcript levels revealed by qRT-PCR of RdDM targets in the AmiGO-RBR mutant background vs the control **a**. The expression levels for every transcript were obtained from biological and technical triplicates, and the data plotted represents the average of these triplicates to which standard deviation was applied. RBR-ChIP and DRM2-mediated DNA methylation common targets **b**. Key exam-

ples of RBR-DRM2 common targets **c**. RdDM proteins that have been validated as RBR direct interactors (green lines) **d**, those that are putatively binding RBR (red lines), gray balloons are PPIs with DRM2 reported in the IntAct database <https://www.ebi.ac.uk/intact/interactions?conversationContext=4>, (Created with BioRender.com)

such as RDM1, AGO4, AGO9, and ZOP1 (Fig. 5d (<https://www.ebi.ac.uk/intact/interactions?conversationContext=4>)).

Our findings and predictions open novel working hypotheses for diverse potential RBR-RdDM interactions (Fig. 5d), including the RBR-DRM2 complex, regulating TEs and interesting lineage-specific transcription factors. Future experimental analyses are needed to show if all the proteins containing the canonical and non-canonical LXCXE motif are truly binding RBR in Arabidopsis, and the regulatory and developmental consequences of such interactions, since there is evidence indicating that the non-canonical SLiM LxCxD is also capable of mediating low-affinity contacts with Retinoblastoma proteins in both animals and plants

(Singh et al. 2005; Palopoli et al. 2018; Ramanujan et al. 2021). Indeed, as both glutamic (E) and aspartic acid (D) residues present in canonical and non-canonical SLiMs, respectively, present negatively charged sidechains it is unlikely that a change in the third position of the SLiM contributes to a change in binding affinity to pRB/RBR.

Other interesting questions to solve are if these PPIs are also conserved along the plant phylogeny and what function they may have in plant clades with strikingly different plant bodies and morpho-physiological characteristics. It has been shown that loss of function mutants in members of the RdDM pathway show phenotypes in diverse developmental processes and stages of Arabidopsis (He et al.

2009; reviewed in Matzke et al. 2015; Mendes et al. 2020). For example, columella phenotypes observed in RdDM and RBR loss-of-function mutants, shown in Figs. 3 and 4 are consistent with findings in this tissue by Kawakatsu et al. (2016), who reported that the *Arabidopsis* columella root cap genome is hypermethylated and transcripts encoding RdDM factors, as well as 24-nt small RNAs (sRNAs), are more abundant in this tissue than any other root cell type.

Another interesting finding in this study is a common set of genes which are bound both by RBR and DRM2. Among the most interesting ones are WUSCHEL and genes involved in cell cycle regulation. It has been reported that in *rbr1-2* mutants, supernumerary megaspore mother cells (MMCs) are formed, a phenotype that correlates with *WUS* transcriptional deregulation Zhao et al. (2017a, b). Indeed, these authors demonstrate that RBR binds to a specific region on the *WUS* promoter. It has also been shown that in the *drm1;drm2;cmt3* *WUS* transcription is de-repressed during root regeneration and that two non-CG sites in the promoter of this gene might be related to *WUS* silencing in *Arabidopsis* roots (Shermer et al. 2015). Recently Mendes et al. (2020) showed that *drm1;drm2* double mutants develop multiple MMCs, a phenotype also described for other mutants in key genes of the RdDM pathway, such as *rdr6* and *ago9* (Olmedo-Monfil et al. 2010).

The putative function of RBR–DRM2 regulating the expression of genes involved in cell cycle progression, which are normally expressed in root meristematic cells, such as *CDC6B* or *CYCA1;1*, may correlate with some of the root phenotypes reported in Figs. 3 and 4. However, root phenotypes in RdDM mutants are not similar in all cases, and such contrasting phenotypes may be caused by the deregulation of hundreds of genes with diverse cellular functions. In this case, future studies are needed to clarify how RBR in concert with the RdDM pathway control gene expression of common targets, and what is the relevance of such regulatory mechanisms not only in root development but also in other organs and tissues, where these PPIs converge.

**Author contributions statement** Conceived the project: AC-R and MA-V. Performed *wetlab* and *in silico* experiments: AC-R, AE-C, JL-R, IB. Analyzed the data: AC-R, MA-V, BS, IB, AE-C and JL-R. Contributed reagents and equipment: BS, MA-V, AC-R. Wrote the manuscript with inputs from all coauthors: AC-R and MA-V.

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**Data availability** All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Declarations

**Conflict of interest** The authors declare no conflicts of interest.

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