Transcriptional repression by MYB3R proteins regulates plant organ growth

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Abstract

In multicellular organisms, temporal and spatial regulation of cell proliferation is central for generating organs with defined sizes and morphologies. For establishing and maintaining the post-mitotic quiescent state during cell differentiation, it is important to repress genes with mitotic functions. We found that three of the *Arabidopsis* MYB3R transcription factors synergistically maintain G2/M-specific genes repressed in post-mitotic cells and restrict the time window of mitotic gene expression in proliferating cells. The combined mutants of the three repressor-type MYB3R genes displayed long roots, enlarged leaves, embryos, and seeds. Genome-wide chromatin immunoprecipitation revealed that MYB3R3 binds to the promoters of G2/M-specific genes and to E2F target genes. MYB3R3 associates with the repressor-type E2F, E2FC, and the RETINOBLASTOMA RELATED proteins. In contrast, the activator MYB3R4 was in complex with E2FB in proliferating cells. With mass spectrometry and pairwise interaction assays, we identified some of the other conserved components of the multiprotein complexes, known as DREAM/dREAM in human and flies. In plants, these repressor complexes are important for periodic expression during cell cycle and to establish a post-mitotic quiescent state determining organ size.

Keywords cell cycle regulation; cell differentiation; DREAM complex; G2/M phase; MYB transcription factors

Subject Categories Transcription; Cell Cycle; Plant Biology

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Introduction

During organ development, cell proliferation and differentiation are regulated in a temporally and spatially coordinated manner. In general, there is a gradual decrease in cell division activity as organogenesis proceeds, and most, if not all, cells eventually stop dividing and differentiate. The scheduled cessation of cell division is critical for the formation of organs with genetically defined sizes and morphologies (Conlon & Raff, 1999; Potter & Xu, 2001; De Vos *et al*, 2012; Hepworth & Lenhard, 2014). Organ initiation and growth in plants are largely post-embryonic that relies almost entirely on the activity of meristems, which contain dividing undifferentiated cells and are maintained throughout the lifetime of plants (Scheres,

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2007). As cells exit the meristematic zone, cell proliferation ceases which relies on the negative regulation of cell cycle progression, but the mechanisms are not fully understood (Gutierrez, 2005; de Jager et al, 2005; Inzé & De Veylder, 2006; Komaki & Sugimoto, 2012).

Leaf and sepal growth is determinate, and these organs represent the most studied models for the temporal and spatial regulation of cell proliferation. At initial stages of organ development, active cell division leads to a rapid increase in the number of cells within the primordia, which is followed by the gradual decrease in cell division activities (Beemster et al, 2005; Roeder et al, 2010; Andriankaja et al, 2012). In *Arabidopsis*, the cessation of cell division is associated with the onset of endoreduplication, in which DNA replication is repeated without intervening mitosis, leading to an increase in cellular DNA content (De Veylder et al, 2011; Fox & Duronio, 2013). As differentiation takes place, cells enter a quiescent state that is typically maintained for the rest of the plant's life. Similar temporal changes in cell division activity occur during the indeterminate growth of the root, generating an apical-basal positional gradient of cell division activity (Vanstraelen et al, 2009; Ivanov & Dubrovsky, 2013).

In *Arabidopsis*, genome-wide expression profiling uncovered the dynamic transcriptional regulation during root and leaf development (Birnbaum et al, 2003; Beemster et al, 2005). The cluster of G2/M-phase-specific genes showed rapid and pronounced downregulation as cells differentiate which was correlated with the cessation of cell division. However, it is not clear whether such downregulation is an active process that is mediated by developmentally regulated transcriptional repression, or whether it is an indirect consequence of decreased cell proliferation activity.

It is widely accepted that transcriptional regulations are essential for the developmental control of cell division (Berckmans & De Veylder, 2009). One of the important mechanisms for such regulation is based on the retinoblastoma (RB)-E2F pathway, which regulates the expression of many genes required for cell proliferation. The conserved tumor suppressor RB, called RB-related (RBR) in *Arabidopsis*, is known to associate with three functionally distinct E2F transcription factors. RBR may repress cell proliferation through E2FB (Magyar et al, 2012), while E2FC acts as transcriptional repressor and is required for the timed cessation of cell division and occurrence of endoreduplication during leaf development (del Pozo et al, 2006; de Jager et al, 2009). Thus, both RBR1 and E2FC act as negative regulators for cell division and are required for scheduled exit from the mitotic cell cycle that might be important to set up the developmental gradient of cell division activities in growing organs.

In mammalian cells, DP1, the RB-related protein p130 and E2F4, together with the MuvB complex (containing LIN9, LIN37, LIN52, LIN54, and RBP4), make a multiprotein complex known as DP, RB-like E2F, and MuvB (DREAM) complex (Litovchick et al, 2007; Sadasivam & DeCaprio, 2013). Current evidence suggests that this complex acts as a repressor of cell cycle-regulated genes during quiescence (Litovchick et al, 2007). When a quiescent mammalian cell is stimulated to enter the cell cycle, the complex releases p130, DP1, and E2F4 and instead recruits a member of the Myb transcription factors, B-MYB, to promoters of G2/M-specific genes. This recruitment is required for transcriptional activation of various genes essential for mitosis (Sadasivam et al, 2012). A similar multiprotein complex called *Drosophila*

RBF, E2F2, and Myb (dREAM) is known in flies, which acts for repression of a variety of developmentally regulated genes and also for activation of the mitotic genes in proliferating cells (Korenjak et al, 2004; Georlette et al, 2007). The latter function of dREAM complex is attributed to dMYB, a single *Myb* gene in *Drosophila* (Beall et al, 2004; Lipsick, 2004). Accumulating evidence suggests that the conserved multiprotein complex has general roles as a global repressor and that Myb proteins counteract the repression (Sadasivam & DeCaprio, 2013). Although plants share the conserved members of E2F, DP, RB, Myb proteins (Vandepoele et al, 2002; Ito, 2005), and some MuvB components, the existence of DREAM/dREAM repressor complex and its possible functions are not known in plants.

There are more than a hundred Myb genes encoded in plant genomes. However, most of Myb proteins in plants contain only two Myb repeats in N-terminal DNA-binding domain, as opposed to the three Myb repeats in animals (Dubos et al, 2010). The plant-specific two-repeat, R2R3-Myb genes have diverse roles in plant development and environmental responses. On the other hand, a small number of three Myb repeat-containing plant proteins, called R1R2R3-Myb or MYB3R, are linked to the regulation of mitosis (Ito, 2005). We have previously shown that plant MYB3R proteins regulate many G2/M-specific genes such as *CYCB1*, *CYCB2*, and *CDKB2*, by binding to the common *cis*-acting elements that are known as MSA element (Ito et al, 1998, 2001; Kato et al, 2009; Haga et al, 2011). In *Arabidopsis*, there are five genes that encode MYB3R transcription factors (Dubos et al, 2010). Our previous studies showed that two structurally related MYB3R proteins, MYB3R1 and MYB3R4, act as transcriptional activators on many, if not on all, G2/M-specific genes (Haga et al, 2011). We also showed that they are required for cytokinesis via the transcriptional activation of a critical target gene, *KNOLLE* (*KN*), a gene essential for cell plate formation (Lukowitz et al, 1996; Haga et al, 2007).

Here, we report on the function of another pair of closely related MYB3R genes, *MYB3R3* and *MYB3R5*, to be repressors of the transcription of G2/M-specific genes. The previously identified activator MYB3R1 can also have redundant repressor functions with *MYB3R3* and *MYB3R5* to inhibit the transcription of many G2/M-specific genes most pronouncedly in differentiated cells that have ceased to proliferate. The triple mutant of these three MYB3R genes shows hyperplasia, generating organs with increased sizes but also some developmental abnormalities and irregular cell divisions during embryogenesis. Genome-wide transcriptional profiling and chromatin immunoprecipitation experiments with MYB3R3 identified G2/M-specific target genes and show that MYB3R3 can also associate with promoters known to be E2F targets. However, the expression of these E2F target genes is not dependent on the repressor MYB3Rs. Accordingly, our biochemical data showed that MYB3R3 associates with E2FC and RBR1, while the activator MYB3R4 is found together with E2FB and RBR1. With mass spectrometry detection and pairwise interaction assays, we could also show other known DREAM/dREAM complex components together with MYB3R3, RBR1, and E2FB, but the exact composition of these complexes remains to be elucidated. We propose that the repressor MYB3R proteins may form complexes that are important for restricting the time window of mitotic gene expression in proliferating cells and for the maintenance of repressed states of G2/M-specific genes in post-mitotic cells.

Results

MYB3R1, MYB3R3, and MYB3R5 act redundantly as transcriptional repressors

Phylogenetic analysis showed that there are two evolutionarily conserved groups in plant MYB3R family (Fig 1A). One contains MYB3R1 and MYB3R4 (hereafter MYB3R1/4) from *Arabidopsis*, which were previously shown to act as transcriptional activators (Ito et al, 2001; Haga et al, 2007, 2011). The other group contains two *Arabidopsis* MYB3Rs, MYB3R3 and MYB3R5 (hereafter MYB3R3/5), whose function was addressed in this study. We analyzed T-DNA insertion alleles of these genes, *myb3r3-1*, *myb3r3-2*, and *myb3r5-1*, all of which resulted in complete loss of normal transcripts (Fig 1B and Supplementary Fig S1). Both MYB3R3 alleles gave identical phenotypes when combined with the other *myb3r* mutants (see below), and thus are hereafter referred to as *myb3r3*.

The *myb3r1/4* double mutant was reported to have aberrant cytokinesis (Haga et al, 2007) suggesting that these two proteins positively regulate mitosis, a function which is not shared with MYB3R3/5, because combined triple and quadruple mutants do not influence these cytokinetic defects (Supplementary Fig S2). To analyze the roles for MYB3R3/5 in transcriptional regulation of G2/M-specific genes, we performed quantitative RT-PCR (qRT-PCR) analyses of seedlings with double *myb3r3/5* mutation (Fig 1C, gray bars) and found significant upregulation of many, but not all of the G2/M-specific genes with MSA element, which include those encoding mitotic regulators, CYCB1;1, CYCB1;2, CDC20.1, and also microtubule-associated proteins with cytokinetic functions, PLEIADE (PLE)/MAP65-3 (Müller et al, 2004) and ENDOSPERM DEFECTIVE1 (EDE1) (Pignocchi et al, 2009). This indicated a repressor function for MYB3R3/5.

We then tested genetic interactions among MYB3Rs for regulating G2/M-specific genes, using qRT-PCR analysis. In the *myb3r1/3/5*

triple mutant, there is a further upregulation of G2/M-specific genes, *EDE1* and *CYCB1;1*, compared to the double *myb3r3/5* but not in the *myb3r3/4/5* (Fig 1D). This raised the unexpected possibility that MYB3R1, but not MYB3R4, has redundant functions both with activator- and repressor-type MYB3Rs. In the *myb3r1/3/5* triple mutant, a large cohort of G2/M-specific genes are further upregulated in comparison to the double *myb3r3/5* as shown by qRT-PCR (Fig 1C, black bars). To gain insight whether this might also be the case on a genome-wide scale for all mitotic genes, we performed microarray expression profiling of seedlings (Supplementary Fig S3). Although this microarray analysis was done with a single biological replicate, the transcriptome data suggested that many genes annotated as “mitotic” or “G2/M-specific” were upregulated in *myb3r1/3/5* seedlings as compared to wild type or the *myb3r3/5* double mutant. It also suggested that this upregulation was specific for the gene sets annotated as “G2/M-specific” and “mitotic”, while genes related to other cell cycle phases were essentially unaffected (Supplementary Fig S4, list of each gene set is found in Supplementary Table S1).

To further investigate the genetic interactions between activator- and repressor-type MYB3Rs on mitotic gene regulation, we analyzed mitotic gene expression profiles in *myb3r* mutant combinations (Supplementary Fig S5, see quantitative expression data of individual mitotic genes in Supplementary Table S2). The single *myb3r1* mutant had little impact on gene expression and *myb3r4* showed minor downregulation, but in the double mutant, the downregulation was enhanced for a cohort of mitotic genes (Supplementary Fig S5). The single *myb3r3* and *myb3r5* mutants also displayed minor effects, but the double *myb3r3/5* mutants showed upregulation for a distinct set of mitotic genes (Supplementary Fig S3). The upregulation of these genes was enhanced by introducing *myb3r1* but not *myb3r4*. In the quadruple *myb3r1/3/4/5* mutant, the upregulation and downregulation of these two cohorts of mitotic genes were combined (Supplementary Fig S5). The qRT-PCR analysis of *EDE1* and *CYCB1;1* in mutant combinations of *myb3r1*, *myb3r3*, and *myb3r5* also confirmed that MYB3R1 can play redundant roles with repressor-type MYB3Rs and showed these repressor-type MYB3Rs act redundantly and contribute differently to the transcriptional repression (Fig 1E). To validate the microarray results obtained from different mutant seedlings, we selected representative genes for up-, downregulated, and unchanged clusters and performed qRT-PCR analysis in biological triplicates (Supplementary Fig S6). This experiment fully confirmed the microarray data and our conclusion that MYB3R1 has redundant functions both with the activator MYB3R4 and the repressor MYB3R3/5. Hereafter, we refer to MYB3R1/3/5 as repressor MYBs (Rep-MYBs) and MYB3R1/4 as activator MYBs (Act-MYBs).

Repression of G2/M-specific genes in post-mitotic cells

To address genetically whether or not the Act-MYB and Rep-MYB act antagonistically in the same cells, we aimed to correlate the expression of a critical mitotic target gene, *KN* and the cytokinetic phenotypes in the quadruple *myb3r1/3/4/5* mutant. We found that the expression of *KN*, which is known to be downregulated in *myb3r1/4* (Haga et al, 2007), but increased in *myb3r1/3/5*, came close to wild-type levels in the quadruple *myb3r1/3/4/5* mutant, but the cytokinetic defect remained (Fig 2A). This argues against that

Act-MYB and Rep-MYB act in the same cell, but rather suggest that their functions are spatially and/or temporally separated. We reasoned that the loss of Rep-MYB might affect the expression of *KN* and other G2/M-specific genes in post-mitotic cells, which would not lead to the rescue of cytokinetic defects caused by the loss of Act-MYB in dividing cells.

To test this hypothesis, we determined at which developmental stages the Rep-MYBs act through time-course expression analysis of G2/M-specific genes during leaf development (Fig 2B and Supplementary Fig S7A). In wild type, the transcript levels of G2/M-specific genes, *EDE1* and *KN*, were initially high at 5 days after germination (DAG), showed rapid decrease by 9 DAG, and stayed at low levels until 15 DAG (Fig 2B). It was clear that *EDE1* and other G2/M-specific genes examined (except for *IMK2*) were upregulated in mature leaves (15 DAG) with stronger effects in the *myb3r1/3/5* triple mutant than in the double *myb3r3/5* mutant (Fig 2B and Supplementary Fig S7A). *KN* expression was not affected by the *myb3r3/5* double and *myb3r1/3/5* triple mutations in proliferating stage of leaves at 5 DAG but was predominately upregulated at later leaf development stages (Fig 2B). Consistent with the lack of phenotypic complementation in *myb3r1/3/4/5* (Fig 2A), expression of *KN* in young leaves was not recovered in the quadruple mutant in comparison with *myb3r1/4* (Supplementary Fig S7B). To see whether cell proliferation is prolonged during development in these mutants, we determined *CYCD3;1* expression, which is known to be correlated with cell division activities (Beemster et al, 2005), but *CYCD3;1* showed age-dependent downregulation comparable with that in wild type (Fig 2B).

To determine genome-wide the cohort of genes that are regulated by Rep-MYB, we performed microarray analysis of leaves at two developmental stages, comparing wild type and the *myb3r1/3/5* triple mutant. The results showed that the loss of Rep-MYB dramatically upregulated most of the G2/M-specific genes in mature leaves (15 DAG; Fig 2C), while the upregulation was less pronounced in young leaves and was specific for a subset of genes, including *EDE1* (5 DAG; Fig 2C), which showed an increased expression both at early and late stages compared to wild type (Fig 2B). In agreement with what we found in seedlings, the de-repression was specific to the genes annotated as “G2/M-specific” and “mitotic”, but not to genes related to other cell cycle phases such as “E2F target”, “replication”, and “histone” (Supplementary Fig S8). Statistical analysis of the microarray data obtained from biological triplicates of the 15-DAG leaf samples identified 72 and 5 genes that are significantly up- and downregulated, respectively, upon loss of Rep-MYB (Supplementary Table S3). The upregulated genes showed enrichment of gene ontology (GO) categories such as “cell cycle”, “cytokinesis,” and “M phase” (Supplementary Fig S9A). Venn diagram analysis of upregulated genes showed a striking overlap with G2/M-specific genes, but not with those related to other phases (Supplementary Fig S9B). These results suggest that Rep-MYB may have a role for selectively repressing G2/M-specific genes, especially in post-mitotic cells during organ development.

To validate the generality of this view, we analyzed various organs at different developmental stages. In all the organs examined, qRT-PCR analysis showed that upregulation of G2/M-specific genes due to the loss of Rep-MYB was more pronounced in organs at later stages of development than in young ones (Fig 2D and Supplementary Fig S10).

Figure 2. MYB3R1, MYB3R3, and MYB3R5 act as repressors in post-mitotic cells during organ development.

A Genetic interactions between repressor and activator MYBs. The frequencies of cytokinesis-defective stomata ($n = 6$) and levels of *KN* transcripts ($n = 3$) were quantified using seedlings (9 DAG) with mutations in either repressor MYBs (Δ Rep), activator MYBs (Δ Act), or both (Δ ActRep). The qRT-PCR data were normalized by the levels of *ACT2* expression. Error bars represent SD.

B Expression of G2/M-specific genes during leaf development. The first leaf pairs were harvested from wild-type, *myb3r3/5*, and *myb3r1/3/5* plants at indicated times after germination and were used for qRT-PCR analysis to determine the transcript levels of *EDE1* and *KNOLLE*. *CYCD3;1* was also analyzed as a control with an expression that is dependent on cell division, but not directly regulated by MYB3Rs. Images of representative wild-type plants at indicated times are also shown, in which the first leaf pairs are indicated by arrowheads.

C Preferential upregulation of G2/M-specific genes in old leaves upon loss of repressor MYBs. Microarray analysis of the first leaf pair was conducted in wild-type and *myb3r1/3/5* plants, and the expression signals are shown as scattered plots where red dots indicate G2/M-specific genes.

D Upregulation of G2/M-specific genes in various organs upon loss of repressor MYBs. Various organs at different developmental stages were harvested and used for qRT-PCR analysis to determine the expression levels of *CYCB1;2* and *EDE1*. The qPCR data were normalized by the levels of *ACT2* expression. Data are shown as fold upregulation in *myb3r1/3/5* compared to wild-type plants. Error bars represent SD for $n = 3$.

Data Information: WT, wild type; $\Delta 3,5$, *myb3r3/5* double mutant; $\Delta 1,3,5$, *myb3r1/3/5* triple mutant.

To determine the developmental position of cells that show this ectopic expression of mitotic genes in the *myb3r1/3/5* triple mutant, we utilized fluorescent protein markers that are driven by promoters of the G2/M-specific genes, *CYCB1;2*, *EDE1*, and *AtNACK1/HINKEL* (Strompen *et al*, 2002). First, we used the *CYCB1;2*-YFP reporter, in which yellow fluorescent protein (YFP) fused to cyclin destruction box is driven by the *CYCB1;2* promoter (Iwata *et al*, 2011). In wild-type plants, *CYCB1;2*-YFP expression is confined to dividing cells in leaf epidermis and root meristems. In triple *myb3r1/3/5* mutants, however, there was a strong YFP expression in differentiated cells, such as large lobbed pavement cells in leaf epidermis (Fig 3A), cells in the elongation zone of roots (Fig 3B), and even in root hair cells (Fig 3C), where *CYCB1;2*-YFP expression is not normally observed. Some YFP-expressing cells had enlarged nuclei, suggesting that the promoter remained active after the onset of endoreduplication (Fig 3A and C, see also Supplementary Fig S11 for leaves at various developmental stages). For the proAtNACK1::YFP and proEDE1::YFP markers, a similar ectopic expression was observed in differentiated non-proliferating cells, including pavement cells and mature guard cells in leaves, and epidermal cells in hypocotyls (Fig 3D

and E, see also Supplementary Fig S12A). Taken together, our data strongly suggest that Rep-MYBs have a critical function in the general maintenance of the repressed state of G2/M-specific genes after the exit from cell proliferation.

Roles of repressor MYBs in proliferating cells

Although mutations in Rep-MYB showed more pronounced upregulation in fully developed organs, we also observed increased transcript levels of some G2/M-specific genes in the developing young organs (Fig 2B–D). To visualize how proliferating cells were affected by the loss of Rep-MYBs, we examined the expression of the *CYCB1;2*-YFP reporter in meristems and developing young organs. In wild-type root meristems, this reporter showed a characteristic patchy pattern of expression that was consistent with its previously reported G2/M specificity (Iwata *et al*, 2011). Compared to wild type, we observed a larger proportion of cells expressing the *CYCB1;2*-YFP marker in root meristems of the *myb3r1/3/5* mutant (Fig 3B). The early heart stage embryo is largely comprised of proliferating cells. In contrast to the typical patchy pattern of expression

Figure 3. Ectopic expression of G2/M-specific genes in proliferating and post-mitotic quiescent cells upon loss of repressor MYBs.

- A Expression of CYCB1;2-YFP in the leaf epidermis of 9-day-old plants. Leaves from *myb3r1/3/5* ($\Delta 1,3,5$) and wild-type (WT) plants were counterstained with propidium iodide for cell walls to visualize cell outlines and were analyzed by confocal microscopy. In *myb3r1/3/5* leaves, CYCB1;2-YFP expression was often observed in cells with enlarged nuclei that had presumably undergone endoreduplication, as indicated by asterisks.
- B Expression of CYCB1;2-YFP in roots of 3-day-old seedlings. CYCB1;2-YFP expression was expanded toward the basal zone of roots in *myb3r1/3/5* seedlings.
- C Ectopic expression of CYCB1;2-YFP in terminally differentiated root hair cells in *myb3r1/3/5* seedlings (asterisks).
- D Expression of proAtNACK1::YFP in epidermal non-dividing cells in *myb3r1/3/5* hypocotyl (asterisks).
- E Ectopic expression of proEDE1::YFP in maturing guard cells in *myb3r1/3/5* leaves (asterisks). Such expression was absent in wild-type leaves (arrowheads).
- F Expression of CYCB1;2-YFP in the developing embryo. In a *myb3r1/3/5* embryo, a greater population of cells expressed CYCB1;2-YFP compared with a wild-type embryo.
- G Expression of CYCB1;2-YFP in cotyledon from 3-day-old seedlings. Asterisks indicate YFP expression in endoreduplicated cells with enlarged nuclei.
- H Quantitative comparison of CYCB1;2-YFP-expressing cells in *myb3r1/3/5* and wild-type plants. Proportion of CYCB1;2-YFP-expressing cells was determined among epidermal cells in root tips of 5-day-old seedlings ($n = 12$), and meristemoids and guard mother cells (GMC) in first leaf pairs of 9-day-old seedlings ($n = 8$). Error bars represent SD. The asterisks in the graphs show differences that are statistically significant (t -test P -value < 0.05).

Data Information: WT, wild type; $\Delta 1,3,5$, *myb3r1/3/5* triple mutant. Scale bars, 50 μ m in (A–E, G), 200 μ m in (F).

in wild type, the CYCB1;2-YFP expression was almost completely uniform in *myb3r1/3/5* mutant (Fig 3F). Similarly, a more uniform expression pattern was observed in proliferating cells of young cotyledons at 3 DAG (Fig 3G) and leaves at 7 DAG in *myb3r1/3/5* mutant (see Supplementary Fig S11). For quantitative comparison of CYCB1;2-YFP expression in *myb3r1/3/5* and wild type, we determined the proportion of YFP-positive cells in epidermal layer of root meristems and in proliferating meristemoids and guard mother cells (GMC) of young developing leaves (Fig 3H). The results show that a

significantly larger proportion of proliferating cells are expressing CYCB1;2-YFP in the *myb3r1/3/5* triple mutant, compared to wild type. Similar uniform expression was also observed for proAtNACK1::YFP and proEDE1::YFP in proliferating cells (Supplementary Fig S12). These data suggest that, in the *myb3r1/3/5* triple mutant, either the CYCB1;2-YFP became ectopically expressed outside of its normal mitotic time window or there is a prolongation of or arrest in G2 phase. Cell cycle analysis by flow cytometry of young leaves and root meristems (see below) showed no evidence of increased G2

DNA content in the *myb3r1/3/5* triple mutant. Therefore, our data are consistent with the idea that Rep-MYB is required to suppress transcription outside of the G2/M phase in proliferating cells.

To genetically demonstrate the functional relevance on the regulation of mitotic genes in proliferating cells, we studied the genetic interactions of *myb3r1/3/5* mutant with weak mutant alleles of *MAP65-3/PLE* and *EDE1*, two genes that encode microtubule-associated proteins expressed specifically at the G2/M phase of the cell cycle and essential for mitosis and cytokinesis (Müller *et al*, 2004; Pignocchi *et al*, 2009). Both genes possess typical MSA motifs in their promoters and are upregulated after the loss of Rep-MYB (Fig 1C and D, and Supplementary Fig S6), but in contrast to *KN*, these genes are upregulated both in proliferating cells and in cells that exited proliferation at late developmental stages as compared to wild type (Fig 2B). Both *ple-2* (Supplementary Fig S13B) and *ede-1* (Supplementary Fig S15A) alleles have mutations at splice sites, producing mis-spliced transcripts that are likely to encode hypoactive proteins (Müller *et al*, 2002, 2004; Pignocchi *et al*, 2009). The introduction of *ple-2* into *myb3r1/3/5* backgrounds led to significant suppression of the cytokinetic defects seen in *ple-2* roots (Fig 4A). The reduced root growth phenotype of *ple-2* was also markedly rescued in the *myb3r1/3/5* mutant (Supplementary Fig S13A). This confirmed the idea that the reduced activity of PLE in the *ple-2* hypoactive mutant was compensated by its increased expression due to the de-repression in the *myb3r1/3/5* mutant. Consistent with this interpretation, we detected the upregulation of *ple-2* incorrectly spliced transcripts upon loss of Rep-MYB (Supplementary Fig S13C). We further showed that increased *ple-2* expression under the control of *CDKA;1* promoter can partially rescue the *ple-2* mutant root phenotype (Supplementary Fig S14A and B) and that the null allele of *ple* could not be rescued by the *myb3r1/3/5* (Supplementary Fig S14C). Similar suppression by the loss of Rep-MYB was observed for the cytokinesis defect in *ede1-1*, which was also significantly recovered in the background of the *myb3r3/5* and *myb3r1/3/5* mutants (Fig 4B, and Supplementary Fig S15C), and this phenotypic recovery was consistent with the upregulation of the *ede1-1* transcript (Supplementary Fig S15B). The observed derepression of mitotic genes in meristematic tissues and the genetic data on the rescue of mitotic defects in proliferating cells when hypoactive alleles were upregulated in the *myb3r1/3/5* mutant together suggest that Rep-MYBs do have roles in proliferating cells as well. Consistent with this notion, genes for Rep-MYBs, *MYB3R1*, *MYB3R3*, and *MYB3R5*, are expressed both in proliferating and maturing stages of leaves, whereas *MYB3R4*, without apparent repressive activity, is expressed specifically in proliferating stage (Supplementary Fig S16).

MYB3R3 associates with promoters of G2/M-specific genes and E2F target genes

Next, we asked whether Rep-MYBs associate physically with the MSA elements of target promoters. To test this *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed using plants expressing a functional MYB3R3-GFP fusion that was driven by its own promoter (Supplementary Fig S17). Using anti-GFP antibodies, we tested binding to the promoters of 8 selected G2/M-specific genes and showed that MYB3R3-GFP was significantly enriched on all promoters that contained MSA motifs (Fig 5A). As a negative control, we examined the *CDKA;1* promoter, where no significant

Figure 4. Genetic interactions between *myb3r1/3/5* and *ple-2* or *ede1-1* reveal the functions of repressor MYBs in proliferating cells.

- A** Cytokinesis defects in *ple-2* roots were partially suppressed by combinational mutations in *MYB3R1/3/5*. The roots of plants with the indicated genotypes were stained at 7 DAG by propidium iodide to visualize cell outlines and were analyzed by confocal microscopy. Magnified views of the boxed area are provided below each image, to show cytokinesis defects, such as gapped cell walls and cell wall stubs (indicated by asterisks).
- B** Cytokinesis defects in *ede1-1* cotyledons were partially suppressed by combinational mutations in *MYB3R1/3/5*. Cotyledons of plants (8 DAG) with the indicated genotypes were fixed, cleared, and observed by DIC microscopy. The *ede1-1* mutation causes incomplete cytokinesis of guard mother cells, producing single-celled stomata (asterisk).

Data Information: WT, wild type; $\Delta 3,5$, *myb3r3/5* double mutant; $\Delta 1,3,5$, *myb3r1/3/5* triple mutant. Scale bars, 50 μm in (A), 20 μm in (B).

enrichment was quantified (Fig 5A). To determine the genes bound by MYB3R3 on the genomic scale, we performed ChIP followed by deep sequencing (ChIP-seq). DNA libraries for deep sequencing

Figure 5. Genome-wide identification of MYB3R3-bound genes *in vivo*.

- A** MYB3R3-GFP associates with promoters of G2/M-specific genes. ChIP-qPCR assays were performed using whole seedlings of MYB3R3-GFP, and the results are shown as the percentage of DNA fragments co-immunoprecipitated with anti-GFP antibody relative to input DNA. Grey and black bars indicate the results from MYB3R3-GFP and wild-type seedlings, respectively. Each measurement was performed twice and produced similar results.
- B** Both G2/M-specific genes and E2F target genes are enriched in MYB3R3-bound chromatin regions. Venn diagram analysis was conducted to compare MYB3R3-bound genes with the indicated gene categories. There are significant overlaps between MYB3R3-bound and G2/M-specific genes, and between MYB3R3-bound and E2F target genes with P -value $< 10^{-15}$ in Fisher's exact test. Numbers represent the gene number in each category.
- C** Enrichment of the MSA and E2F motifs in MYB3R3-bound chromatin regions. Enriched sequences in immunoprecipitated DNA fraction were searched using motif-finding software MEME-ChIP web tool (<http://meme.nbcr.net/meme/tools/meme-chip>).
- D** Peak distributions around *CYCB1;2* and *ORC1* genes in the ChIP-seq analysis of MYB3R3-GFP plants. *CYCB1;2* and *ORC1* genes are shown as the representative examples of G2/M-specific and E2F target genes, respectively.

were generated from the immunoprecipitated DNA fraction (ChIP DNA) and input DNA fraction and analyzed by Illumina Genome Analyzer IIx (Supplementary Fig S18A). This analysis identified 398 genes that were significantly enriched in ChIP DNA compared with input DNA fraction (Supplementary Tables S4 and S5). This set of genes significantly overlapped with the upregulated genes in *myb3r1/3/5* (Supplementary Fig S18B) and showed extensive enrichment of GO categories such as “cell cycle”, “DNA replication” as well as “cytokinesis” (Supplementary Fig S18C, Supplementary Table S5). Venn diagram analysis showed significant overlaps not only with G2/M-specific genes as expected, but also with E2F target

genes (Fig 5B). Accordingly, we found that the ChIP DNA significantly overrepresented both E2F-like and MSA-like motifs (Fig 5C, Supplementary Fig S18D). In most cases, binding of MYB3R3 was observed around transcriptional start sites as expected from the positions of the MSA elements (Fig 5D).

MYB3R3 and MYB3R4 are detected in complex with RBR1 and different E2F isoforms

Our ChIP-seq analysis showed association of MYB3R3 with E2F target genes, which indicated the presence of DREAM/dREAM-like

complex in plants. To begin to investigate the existence of this complex, we performed pull-down experiments with available MYB3R3-GFP, RBR1-GFP, and E2FB-GFP plants using anti-GFP antibody and detected associated proteins with mass spectrometry (Supplementary Fig S19). We could find conserved components of the MuvB core of the plant DREAM/dREAM-like complex; ALY2, ALY3 and TCX5 with MYB3R3-GFP, and some of these were also present with RBR1-GFP and E2FB-GFP (see Supplementary Table S6 for the list of *Arabidopsis* homologs of DREAM/dREAM complex components). In these experiments, we could not find evidence for the association of MYB3R3 with RBR1, E2Fs, or DPs.

To address whether this is indeed the case, we used a more sensitive detection method of co-immunoprecipitation (Co-IP) assays using the MYB3R3-GFP plants. Immunoprecipitates were subjected to Western blot analysis with antibodies specific to RBR1, E2FB (Fig 6A, see Supplementary Fig S20A for antibody specificity of E2FB), and E2FC (Fig 6C). We found that MYB3R3-GFP did associate with RBR1 and E2FC, but not with E2FB (Fig 6A and C, see also Supplementary Fig S20B and C). These associations were dependent on the developmental stage of leaves; MYB3R3-GFP exists in complex

with RBR1 in both earlier and later stages (8 and 14 DAG) (Fig 6A), whereas E2FC was only detected in the complex at later stages (15 DAG) of leaf development (Fig 6C). The GFP-specific antibody repeatedly recognized two bands in Co-IP samples of MYB3R3-GFP, one of which (around 80 kDa) that corresponds with the calculated molecular weight of the fusion protein, and a faster migrating form that may represent a degradation product of MYB3R3-GFP (Fig 6A).

Next, we conducted similar Co-IP assays for analyzing GFP-MYB3R4, which belongs to Act-MYB (Fig 6B). The GFP-MYB3R4 was found to interact with RBR1, but unlike MYB3R3-GFP, it associates with E2FB and only at an early stage of leaf development (Fig 6B, see also Supplementary Fig S20C–E), but not with E2FC at any stages (Fig 6C). Our data suggest that the repressor MYB3R3 and activator MYB3R4 may exist in different complexes that contain different E2F isoforms with distinct functions and dynamic properties during development. In the Co-IP experiments, anti-GFP antibody could only detect MYB3R3-GFP and GFP-MYB3R4 when enriched in Co-IP samples, but not in crude extract (Fig 6A and B), suggesting the low abundance of these proteins expressed under the control of their native promoters.

Figure 6. MYB3R3 and MYB3R4 both interact with RBR1 and differently associate with E2F isoforms.

A, B MYB3R3-GFP and GFP-MYB3R4 both interact with RBR1 and CDKA;1, but with a different E2F isoform in *Arabidopsis* leaves. IP was performed with anti-GFP antibodies from protein extracts prepared from first leaf pairs of MYB3R3-GFP or GFP-MYB3R4 transgenic plants at indicated days after germination (DAG). In these transgenic plants, expression of GFP fusion proteins was driven by the corresponding native promoters. Co-IP of RBR1 and E2FB was examined by Western blot analyses using corresponding antibodies. For detection of MYB3R3-GFP and CDKA;1, anti-GFP and anti-PSTAIR (specific to CDKA;1) antibodies were used. As input, 1/10 of IP was loaded. Coomassie staining of the same membrane was used as a loading control.

C MYB3R3-GFP interacts with E2FC, but GFP-MYB3R4 does not. IP was performed with anti-GFP antibodies from protein extracts prepared from first leaf pairs of MYB3R3-GFP or GFP-MYB3R4 transgenic plants at indicated days after germination (DAG). Co-IP of E2FC and CDKA;1 was examined by Western blot analyses using anti-E2FC and anti-PSTAIR antibodies, respectively. As input, 1/16 of IP was loaded. Coomassie staining of the same membrane was used as a loading control.

We previously showed that CDKA can regulate activator MYBs (Araki *et al*, 2004). Therefore, we tested whether CDKA can be recruited to the MYB3R3-GFP or GFP-MYB3R4 complexes. We found that CDKA;1 was present with GFP-MYB3R4 at earlier and with MYB3R3-GFP at later stages of leaf development (Fig 6A and B).

To gain a third independent line of evidence for the existence of the DREAM-like complex in *Arabidopsis*, we synthesized the conserved *Arabidopsis* DREAM complex proteins by *in vitro* translation using wheat germ extracts and tested their pairwise interactions with *in vitro*-translated MYB3R3 and MYB3R4 proteins by luminescence proximity AlphaScreen assays (Takahashi *et al*, 2009). The repressor MYB3R3 showed clear interactions with the tested DREAM components, while the luminescence signal was much weaker with activator MYB3R4 (Supplementary Fig S21). Because the *in vitro*-synthesized test proteins were present together with endogenous wheat germ proteins in this assay, the interaction might not be direct, but use bridging proteins, or post-translational modifications provided from the wheat germ extract. Wheat germ cells are essentially post-mitotic, which might explain the difference in supporting complex formation around MYB3R3 but less with MYB3R4.

Taken together, the mass spectrometry data, the co-immunoprecipitation experiments, and the pairwise interaction assays in wheat germ extract show the existence of distinct DREAM-like complexes in proliferating and differentiated plant cells.

Loss of repressor MYBs led to increased organ growth

Derepression of mitotic regulators in the *myb3r1/3/5* triple mutant may result in overproliferation or ectopic cell divisions. In line with this idea, the seeds (Fig 7A) and embryos (Fig 7B) of *myb3r1/3/5* mutant are larger compared to the wild-type control. To study the reasons behind increased sizes, we examined in more details leaf development. Leaves were initiated at around the same time and had similar sizes at the earliest stage of 4 DAG but became visibly larger in the *myb3r1/3/5* mutant than those of wild-type counterpart by 5 DAG (Fig 7C). At 7 DAG, *myb3r1/3/5* leaves contain 9.1% more cells compared with wild-type leaves, while cell sizes were not different, suggesting that the initial size

increase in *myb3r1/3/5* leaves is due to hypertrophy (Fig 7D). To gain further insights into the processes leading to increased leaf growth, we quantified leaf growth parameters by kinematic analysis from the earliest stage (6 DAG onwards) (Beemster *et al*, 2005). We confirmed the initial increase in cell number at the earliest time point, but there was little difference in the cell division rates during the time window of 6–17 DAG between *myb3r1/3/5* and wild-type plants (Supplementary Fig S22A). Difference in cell size was apparent after 9 DAG, with significantly larger mesophyll cells in *myb3r1/3/5* leaves (Supplementary Fig S22A). As a result, *myb3r1/3/5* seedlings attained significantly larger leaves that contained more cells with greater sizes as compared to wild type (Supplementary Fig S22A and B). There was only minor difference in the onset of and progression through endoreduplication between *myb3r1/3/5* and wild type, somewhat decreased ploidy levels throughout leaf development, most prominently at 15 DAG (Fig 7E and F, and Supplementary Fig S23).

The growth of primary roots was also significantly enhanced in the *myb3r1/3/5* mutant (Fig 7G and Supplementary Fig S22C). We also quantified root growth by kinematic analysis and found an elevated cell production rate as cells progress toward the elongation zone where cells exit proliferation in the root meristem (Supplementary Fig S22D). In agreement, *myb3r1/3/5* roots had increased size of meristems (Fig 7H and Supplementary Fig S22F), suggesting a delayed exit from cell proliferation. Flow cytometry measurements in root meristematic zones showed significant reduction in the proportion of cells with 4C DNA content in *myb3r1/3/5* mutant, which is consistent with decreased duration of G2 phase (Supplementary Fig S22E). The formation of lateral roots was also significantly enhanced in *myb3r1/3/5* seedlings (Fig 7G, see also Supplementary Fig S22G). In conclusion, Rep-MYBs function to negatively regulate organ growth, mainly by inhibiting cell proliferation.

In addition to enhanced growth, the *myb3r1/3/5* triple mutants exhibited ectopic cell divisions during embryo development. The suspensors, which connect the embryo proper to maternal tissues, normally comprises a single file of 6–8 cells and is derived from the basal daughter cell of the zygote that undergoes a few rounds of horizontal divisions. In most of the *myb3r1/3/5* embryos, however,

Figure 7. Loss of repressor MYBs causes enhanced organ growth and ectopic cell division.

- A Increased seed size of *myb3r1/3/5* triple mutant. The average seed area after imbibition was determined ($n = 25$).
- B Enlarged embryo of a *myb3r1/3/5* triple mutant.
- C Enhanced leaf growth at the initial stage of leaf development in *myb3r1/3/5* triple mutant. SEM images of initiating first leaf pairs at 4 and 5 DAG are shown. A graph shows the average length of initiating leaves of wild-type and *myb3r1/3/5* seedlings ($n = 6$).
- D Increased cell number causes greater leaf size in *myb3r1/3/5* triple mutants at the early seedling stage. Images show whole seedlings of wild-type and *myb3r1/3/5* plants at 7 and 9 DAG. First leaf pairs are indicated by arrowheads. Graphs show that, at 7 DAG, the difference in the number of palisade cells per leaf, but not their size, is statistically significant ($n = 5$).
- E Time-course changes of ploidy levels in first leaf pairs of wild-type and *myb3r1/3/5* seedlings. The levels of ploidy are expressed by the value called “endoreduplication index,” which represents the average number of endoreduplication cycles that were experienced by the cells ($n = 5$).
- F Ploidy distribution of first leaf pairs at 15 DAG. Representative results of flow cytometric analysis (left) and ploidy distributions (right) of wild-type and *myb3r1/3/5* plants are shown ($n = 5$).
- G Root phenotypes in wild-type and *myb3r1/3/5* plants. Images show seedlings at 6 DAG.
- H Increase in meristem size in *myb3r1/3/5* roots. Images show propidium-iodide-stained roots at 5 DAG. Arrowheads indicate the basal end of the root meristem, which was determined by the position at which the cells start elongating.
- I Ectopic cell division during *myb3r1/3/5* embryo development. A cell clump was formed in suspensor of *myb3r1/3/5* embryo (asterisk).
- J An ectopic shoot apical meristem generated in a *myb3r1/3/5* seedling.

Data Information: WT, wild type; $\Delta 1,3,5$, *myb3r1/3/5* triple mutant. Scale bars: 500 μm in (A) and (B), and 100 μm in (C), (H), and (I). In all panels, error bars represent SD. The asterisks in the graphs show differences that are statistically significant (t -test P -value < 0.05).

we observed irregularly oriented division planes, which created multiple files of cells or clumps of cells, within the suspensor (Fig 7I and Supplementary Fig S22H). Albeit at a very low frequency, we also observed the generation of ectopic meristems, which may have been due to unscheduled division of differentiated cells (Fig 7J). Therefore, Rep-MYBs may also contribute to the maintenance of the quiescent state of post-mitotic cells in different developmental contexts.

Discussion

In plants, populations of rapidly dividing cells are restricted to areas of the apical and lateral meristems and organ primordia. Strikingly, we showed that plants have mechanisms to restrict the expression of mitotic genes to meristematic tissues by the active and continuous repression of these genes outside the meristems. We identified the R1R2R3-Myb proteins, MYB3R1/3/5, to be

Figure 7.

required for the stable repression of G2/M-specific genes during plant development. Loss of Rep-MYB resulted in the upregulation of G2/M-specific genes that was most pronounced in cells that exited proliferation in organs at later stages of development. The global and long-term transcriptional repression of cell proliferation genes in differentiated cells might be important to maintain quiescence during organ development.

In meristematic cells, the loss of Rep-MYB led to nearly uniform expression of G2/M-specific genes in all cells, rather than the normal patchy pattern due to G2/M-phase-restricted expression. This was not due to an arrest in G2 phase, and therefore indicated that in proliferating cells, Rep-MYBs narrow the expression window of their target genes by inhibiting promoter activities outside the mitotic time window. We suggest that the G2/M-phase-specific transcription in plant cells is achieved by the interplay of G2/M-phase-specific promoter activation mediated by the Act-MYBs, and repression by Rep-MYB along the cell cycle except G2/M phase (Supplementary Fig S24).

One of the Rep-MYBs, MYB3R1, did not conform to the neat separation of activator and repressor functions. In the *myb3r1* single mutant, the expression of mitotic genes were largely unaffected, but combining *myb3r1* mutant with *myb3r4* led to downregulation of G2/M-specific genes confirming our previous reports (Haga *et al.*, 2007, 2011), while with *myb3r3/5* to their upregulation (Supplementary Figs S5 and S6). These genetic data indicated that MYB3R1 can act redundantly both with activator and with repressor MYB3Rs, but this might happen in developmentally distinct cells or time window of the cell cycle. This is consistent with the idea that Rep-MYBs including the MYB3R1 have broad expression domains and play roles in differentiated cells and proliferating cells outside the G2/M phase, whereas the Act-MYB, MYB3R4, is exclusively expressed in mitotic cells and solely function during the G2/M phases. Thus, activators and repressors may work in coordination rather than in competition with each other. There are other reports of transcription factors that act both as activators and repressors. For example, the auxin response factors, ARF5, activates transcription of target genes in the presence of auxin, while in the absence it is involved in transcriptional repression by binding to AUX/IAA family proteins that are able to associate with TOPLESS corepressors (Tiwari *et al.*, 2004; Szemenyei *et al.*, 2008). Dual functionality of MYB3R1 may also be provided by the dynamic changes in the composition of protein complexes during the cell cycle or during plant development.

We have previously shown that mutations in Act-MYBs result in the downregulation of many, but not all G2/M-specific genes (Haga *et al.*, 2011). Similarly, here, we found that most, but not all G2/M-specific genes were upregulated when Rep-MYBs were mutated. We noticed that the genes upregulated by the loss of Rep-MYB were largely unaffected by the loss of Act-MYB and conversely, genes that were downregulated by the loss of Act-MYB were unaffected by the loss of Rep-MYB (Supplementary Fig S5). There is no apparent difference in the core MSA sequences between downregulated and unaffected genes in *myb3r1/4* (Haga *et al.*, 2011), and between upregulated and unaffected genes in *myb3r1/3/5* (Supplementary Fig S25). Therefore, these differential sensitivities to the presence or absence of Act-MYBs or Rep-MYBs might depend on the context of promoters of individual G2/M-specific genes. Depending on the

promoter contexts, some genes may be mainly regulated by repression outside the G2/M phase, whereas others may be regulated through activation at the G2/M phase.

We found that the loss of Rep-MYB was associated with hypertrophy both during embryonic and post-embryonic development. The enlargement of leaves at the earliest proliferative growth stage was traced to increased cell numbers soon after leaf emergence. Overproliferation was previously reported in plants with reduced expression of the cell cycle repressors, E2FC and RBR1 (del Pozo *et al.*, 2006; Borghi *et al.*, 2010). Other similarities between *myb3r1/3/5* mutant and the *E2FC* knockdown seedlings include the increased lateral root formation and upregulation of the *CYCB1;1* gene (del Pozo *et al.*, 2006). However, unlike *myb3r1/3/5* mutants, overall organ growth was decreased in the *E2FC* and *RBR1* knockdown plants, mainly due to reduced cell sizes. However, the recently identified *samba* mutant shows abnormalities that closely resemble the *myb3r1/3/5* phenotype of increased organ size, enlarged mature embryos, enhanced root growth, and increased number of lateral roots (Eloy *et al.*, 2012). It should also be noted that both *myb3r1/3/5* and *samba* mutants have elevated numbers of cells as well as increased cell size in leaves. *SAMBA* encodes an activator protein of the anaphase-promoting complex/cyclosome (APC/C), which is an evolutionarily conserved ubiquitin–ligase complex that has a critical function in the degradation of mitotic regulators, including cyclins of CYCB and CYCA classes. Thus, the commonalities between the *myb3r1/3/5* and *samba* mutants in respect to enlarged organ size might be due to the elevated levels of mitotic regulators either through their increased transcription or their decreased degradation, respectively. Correspondingly, it was reported that overexpression of *CYCB1;1* can increase lateral root growth (Doerner *et al.*, 1996). Our results further underline that cell cycle regulation at the G2/M phase can have a strong impact on plant growth and morphogenesis.

The conserved DREAM/dREAM repressor complexes have important roles in the coordination of cell proliferation with the developmentally imposed quiescence and the periodic expression of cell cycle genes in animal cells (Sadasivam & DeCaprio, 2013). We could identify by mass spectrometry the presence of orthologous proteins; LIN54 and LIN9 named as TCX5 and ALY2, ALY3 in *Arabidopsis* in association with both MYB3R3 and RBR1 or E2FB, suggesting that the MuvB core of the DREAM/dREAM-like complex might also link the RBR-E2F and MYB3R functions in *Arabidopsis*. We also confirmed the interaction of MYB3R3 with RBR1 and specifically with E2FC while MYB3R4 with RBR1 and E2FB. Pairwise interaction assays in wheat germ extracts of *in vitro* translated MYB3R3 with conserved plant components of the DREAM/dREAM further support the existence of similar complexes in *Arabidopsis*. Analogous to the roles of Rep-MYBs on mitotic genes in *Arabidopsis*, the mammalian DREAM complexes are required for repression of many cell cycle-regulated genes during quiescence (Litovchick *et al.*, 2007). However, in mammalian cells, B-MYB is only recruited to the DREAM complex at the cell cycle entry, which coincides with the release of the repressive E2F4, DP1, and the Rb-related p130, leading to the activation of G2/M-specific genes (Sadasivam *et al.*, 2012). In contrast, we showed that, in differentiated plant cells, the Rep-MYB can coexist in the same protein complex with RBR1 and/or E2FC. In this respect, this is more similar to the *Drosophila* dREAM complex formed around RBF, E2F2, and Myb (Korenjak *et al.*, 2004;

Georlette *et al.*, 2007). The only Myb protein in *Drosophila*, dMYB, is required both for the transcriptional activation and for the repression (Georlette *et al.*, 2007). In *Arabidopsis*, there are two distinct classes of Myb proteins: Act-MYBs responsible for activation in mitotic cells, while Rep-MYBs acting as global repressors in post-mitotic cells and during the cell cycle progression outside mitosis. Unlike animal Myb proteins that generally act as activators by antagonizing the DREAM/dREAM complexes (Georlette *et al.*, 2007; Sadasivam *et al.*, 2012; DeBruhl *et al.*, 2013), Rep-MYBs may act exclusively as repressors on mitotic genes in *Arabidopsis*. Moreover, our data suggested that *Arabidopsis* has separate complexes containing either the repressor MYB3R3 and E2FC or the activator MYB3R4 and E2FB. Thus, the *Arabidopsis* MYB3R complexes may differ from the conserved animal DREAM/dREAM complexes in terms of their composition and heterogeneity. Further biochemical characterization of the dynamic changes in protein composition of the plant DREAM/dREAM-like complexes during development and cell cycle progression, and genetic studies of the constituents may provide new insights into the molecular basis and the plant-specific nature how cell proliferation is actively restricted to meristems and how the quiescent state is maintained during post-mitotic organ development.

Materials and Methods

Plant materials and growth condition

Arabidopsis thaliana Columbia (Col) was used as the wild-type plant. All mutants and transgenic lines used in this study were in a Col background. The mutant alleles *myb3r3-1* (SALK_041111), *myb3r3-2* (GABI 546A07), and *myb3r5-1* (SALK_031972) were identified from the SALK and GABI-KAT T-DNA collections (Alonso *et al.*, 2003; Rosso *et al.*, 2003) and were used to generate multiple mutant combinations. Other mutant and transgenic lines, *myb3r1-1*, *myb3r4-1*, *ede1-1*, *ple-2*, CYCB1;1-GUS, CYCB1;2-YFP, RBR1-GFP, and E2FB-GFP, were described previously (Colón-Carmona *et al.*, 1999; Müller *et al.*, 2002; Haga *et al.*, 2007; Pignocchi *et al.*, 2009; Iwata *et al.*, 2011; Magyar *et al.*, 2012). The sterilized seeds were germinated on one-half-strength Murashige and Skoog (1/2 MS) medium containing 2% (w/v) sucrose and 0.6% (w/v) agar. Plants were grown on 1/2 MS agar medium or soil under continuous light at 22°C. For the analysis of root phenotypes, plants were grown on a vertical surface of 1/2 MS medium containing 1.0% (w/v) agar.

Plasmid construction and transformation

For the construction of proAtNACK1::YFP and proEDE1::YFP, upstream regions with a length of 2.1 and 1.1 kb, respectively, were amplified by PCR from wild-type (Col) genomic DNA and cloned into the pENTR/D-TOPO vector (Invitrogen). The upstream fragments were then transferred into the pBGYN binary vector (Kubo *et al.*, 2005) using LR Clonase II (Invitrogen), to create the fusion constructs between promoters and nuclear-localized YFP. To create the MYB3R3-GFP expression construct, a genomic fragment of MYB3R3 containing 1.3 kb of upstream region and the complete coding sequence (CDS) was amplified by PCR from Col genomic DNA and cloned into the pENTR/D-TOPO vector to create

pENTR-MYB3R3. The GFP fragment was prepared by PCR and inserted into the pENTR-MYB3R3 plasmid using the In-fusion cloning system (Clontech), to generate an MYB3R3-GFP fusion construct in which GFP is fused in frame to the C-terminus of MYB3R3 in the native genomic context. The fusion construct was then transferred using LR Clonase II to the pGWB501 destination vector (Nakagawa *et al.*, 2007), to create proMYB3R3::MYB3R3-GFP. proMYB3R4::GFP-MYB3R4 was similarly prepared using MYB3R4 genomic fragment containing 1.5 kb upstream region. The primers used for the construction of these plasmids are listed in Supplementary Table S7.

RNA extraction and qRT-PCR

Extraction of total RNA and synthesis of first-strand cDNA were performed as described previously (Haga *et al.*, 2007). Real-time qRT-PCR was performed using the SYBR Premix Ex Taq (Perfect Real Time) Kit (TaKaRa Biomedicals) on a LightCycler480 machine (Roche Diagnostics). See Supplementary Table S7 online for a list of the primers used for qRT-PCR.

Microarray analysis

Microarray analyses were performed using an ATH1 GeneChip® (Affymetrix). A 10-μg aliquot of total RNA was reverse-transcribed, labeled with the One-Cycle Target Labeling and Control Reagents Kit (Affymetrix), and used for hybridization to the chip according to the supplier's protocol. Data analysis was performed using Microarray Suite ver. 5 (Affymetrix) and GeneSpring 7.1 (Agilent Technologies). Per-chip normalization was performed using the 50th percentile of all measurements, to adjust total signal intensity in each chip. For transcriptome profiling in various *myb3r* mutant combinations, whole seedlings at 9 DAG were analyzed with single biological replicate. To compare transcript levels in leaves from 15-day-old plants, three biological replicates for wild type and *myb3r1/3/5* were analyzed. Genes with a false discovery rate (FDR) < 0.05 were defined as upregulated genes in *myb3r1/3/5* compared with wild-type plants. Heat maps of G2/M-specific genes were created using log2-transformed values of fold change levels that were calculated from the normalized data.

Microscopic observation

Microscopic observations with differential interference contrast (DIC) and fluorescent optics were as described previously (Haga *et al.*, 2007). Confocal microscopy of live tissues was carried out using an FV1000 Olympus Confocal Microscope. To counterstain cell outlines, tissues were placed in a solution of 10 μM FM4-64 (Molecular Probes) or 0.1 mg ml⁻¹ propidium iodide for 1–2 min. GFP was excited at 473 nm and fluorescence was detected at 485–545 nm, and FM4-64 and propidium iodide were excited at 559 nm and fluorescence was detected at 570–670 nm.

Clearing of plant materials and histochemical GUS assay were performed as described previously (Haga *et al.*, 2007, 2011).

For scanning electron microscopy (SEM), seedlings were fixed overnight in FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol) at 4°C. The samples were treated with 50, 70, 90, and 100% ethanol and were dried with a critical point drier (HCP-2,

Hitachi, Tokyo, Japan). They were coated with Pt-Pd in an ion sputter (E-1030, Hitachi) on an aluminum SEM sample holder and then observed by SEM (S-3000N, Hitachi).

Other phenotypic analyses

Ploidy analysis was performed as described previously (Haga *et al*, 2011). Briefly, nuclei were extracted from whole leaves using the High Resolution Kit for Plant DNA (Partec). After filtration, nuclei were stained by adding 4',6-diamidino-2-phenylindole (DAPI) solution and analyzed using a PAS flow cytometer (Partec) according to the supplier's instructions. The proportion of nuclei of each ploidy level was estimated as described previously (Imai *et al*, 2006). The endoreduplication index, which represents the average number of endoreduplication cycles per nucleus, was calculated as described previously (Lammens *et al*, 2008).

To measure seed size, photographs were taken under a stereomicroscope (MZ16, Leica), and seed area was measured using the ImageJ software. The sizes of root meristems were determined as described previously (Takahashi *et al*, 2013).

ChIP analysis

The ChIP-qPCR assay was performed as described previously (Nakamichi *et al*, 2010). Briefly, the wild-type and transgenic plants carrying proMYB3R3::MYB3R3-GFP were grown for 9 days in liquid 1/2 MS medium supplemented with 2% (w/v) sucrose, with gentle agitation. Approximately 0.8 g (FW) of whole seedlings were fixed and used in the ChIP assay. An anti-GFP antibody (ab290; Abcam) was used for immunoprecipitation of chromatin complexes bound to MYB3R3-GFP. The amount of each precipitated DNA and input DNA was determined by real-time PCR using specific primers (Supplementary Table S7).

For ChIP-seq analysis, 3 g of MYB3R3-GFP-expressing plants at 9 DAG were fixed as in ChIP-qPCR analysis. Isolation of nuclei, ChIP with anti-GFP antibody, and library construction were performed as described previously (Nakamichi *et al*, 2012). The resulting ChIP and input DNA libraries were sequenced with an Illumina Genome Analyzer IIx (GAIIx).

ChIP-seq data analysis

Basecalls of sequence reads were done by the Illumina GA II pipeline. To map sequence reads on the *Arabidopsis* genome, ChIP and input DNA sequence data in the FASTQ format were analyzed by Bowtie (Langmead *et al*, 2009) against the reference genome TAIR10. Peaks significantly appearing in ChIP DNA compared to the input ($\text{FDR } q < 10^{-50}$) were annotated as binding loci of MYB3R3 (a total of 398 loci) by Model-based Analysis of ChIP-Seq (MACS2) (Zhang *et al*, 2008). MACS2 was also used for validating forward- and reverse-peak distribution. The data for MACS2 were drawn by R (<http://www.r-project.org/>). Peaks from forward and reverse strands were within 200 bp, indicating that DNA fragment sizes in the ChIP library were acceptable and that MYB3R3 associates within a very close region of these peaks (Supplementary Fig S18A). Mapping reads (bam format from CLC-bio data analysis) were visualized with Integrative Genomics Viewer (<http://www.broadinstitute.org/igv/>).

Co-IP experiments

For Co-IP experiments, total protein was extracted from the first leaf pairs of MYB3R3-GFP or GFP-MYB3R4 lines according to Henriques *et al* (2010). Equal amount of proteins (between 400 and 800 μg) was precipitated by using 10 μl of GFP-Trap coupled to magnetic beads (ChromoTek). Immunoprecipitated proteins were eluted from the magnetic beads by SDS-sample buffer and separated on 10% SDS-PAGE together with equal loading of 20–25 μg of total protein extract as input material and blotted to PVDF membrane. Specific antibodies against RBR1 (Horvath *et al*, 2006), E2FB (Magyar *et al*, 2005), E2FC (López-Juez *et al*, 2008), CDKA1 (anti-PSTAIRE, Sigma), and GFP (Roche) were used in immunoblotting experiments.

Data deposition

The microarray and ChIP-seq data have been submitted to Gene Expression Omnibus and assigned to the identifier accession: GSE52298 and GSE60554, respectively. The data can be viewed from the following Web site: <http://www.ncbi.nlm.nih.gov/geo>.

Supplementary information for this article is available online:

<http://emboj.embopress.org>

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Author contributions

KK, ToS, and MI generated plants with multiple mutations in *MYB3R* genes and performed their genetic and molecular characterizations. TD and MO performed microarray analysis. NN, TaS, and TH conducted ChIP experiments and data processing. EI, PC, and MU contributed to the phenotypic characterization of *myb3r1/3/5*. TI and KS generated and analyzed transgenic plants expressing MYB3R3-GFP and GFP-MYB3R4. JHD provided the *ede1-1* mutant and contributed to its genetic characterization. SM and MTH provided the *ple-2* mutant and contributed to its genetic characterization. AI and HH performed kinematic analysis of roots. ZM, TL, and LB performed Co-IP experiments. APS and ZD conducted mass spectrometry analysis. MN and YT contributed to the Alphascreen interaction assays. KK, ToS, LB, and MI wrote the manuscript with contributions from all coauthors.

Conflict of interest

The authors declare that they have no conflict of interest.

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