

Proliferation and cell fate establishment during *Arabidopsis* male gametogenesis depends on the Retinoblastoma protein

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The Retinoblastoma (Rb) protein is a conserved repressor of cell proliferation. In animals and plants, deregulation of Rb protein causes hyperproliferation and perturbs cell differentiation to various degrees. However, the primary developmental impact of the loss of Rb protein has remained unclear. In this study we investigated the direct consequences of Rb protein knockout in the *Arabidopsis* male germline using cytological and molecular markers. The *Arabidopsis* germ line derives from the unequal division of the microspore, producing a small germ cell and a large terminally differentiated vegetative cell. A single division of the germ cell produces the 2 sperm cells. We observed that the loss of Rb protein does not have a major impact on microspore division but causes limited hyperproliferation of the vegetative cell and, to a lesser degree, of the sperm cells. In addition, cell fate is perturbed in a fraction of Rb-defective vegetative cells. These defects are rescued by preventing cell proliferation arising from down-regulation of cyclin-dependent kinase A1. Our results indicate that hyperproliferation caused by the loss of Rb protein prevents or delays cell determination during plant male gametogenesis, providing further evidence for a direct link between fate determination and cell proliferation.

male germline | pollen | cell cycle

In multicellular organisms, cell proliferation and cell differentiation are tightly coordinated both spatially and temporally. One key coordinator is the Rb-E2F pathway (1, 2). As the first identified tumor suppressor gene (3), Rb encodes the retinoblastoma (Rb) protein, which controls cell cycle progression from G1 into S phase (4). Upon phosphorylation by cyclin-dependent kinases (Cdks) at late G1 stage, the Rb protein loses its binding affinity for E2F family transcription factors. The released E2F transcription factors activate downstream cell cycle genes and commit cells to S phase. The Rb protein not only binds to E2F to repress transcription, but also recruits chromatin remodeling factors (5–10). Thus, the Rb protein exerts a broad range of cellular functions beyond cell cycle control, including differentiation (11), senescence (12), and apoptosis (13). Rb^{-/-} knockout mice die from abnormal placenta development (14, 15). Mammalian genomes encode 2 other proteins related to the Rb protein: p107 and p130 (16–18), which further complicates the dissection of Rb function in mammals. It still remains unclear how the Rb protein coordinates cell proliferation and differentiation in animals (16).

In plants, the Rb-E2F pathway is conserved (19). The maize (*Zea mays*) genome contains 3 Rb genes, as in mammals (20–22). *Arabidopsis thaliana* contains a single Rb gene (*RBR*) (23). Loss of function of *RBR* completely impairs female gametogenesis, which precludes direct assessment of the role of the Rb protein in post-embryonic development (24, 25). Loss of *RBR* during female gametogenesis causes over-proliferation but does not appear to have a major effect on cell fate (25–28). To understand the role of *RBR* in development, different inducible systems disrupting *RBR* expression or over-expressing *RBR* were devel-

oped. Virus-induced gene silencing of NbRBR in Tobacco (*Nicotiana benthamiana*) caused deregulation of cell proliferation, differentiation, and endo-reduplication (29). RNA interference and inducible over-expression of *Arabidopsis RBR* impaired stem cell maintenance in roots (30). Inducible expression of a geminivirus RBR-binding protein in *Arabidopsis* leaves suggested that *RBR* prevents cell division and endoreduplication in a cell type-dependent manner (31).

As *RBR* represses *MET1* expression (27) and likely recruits members of chromatin modifying complexes, the loss of *RBR* is expected to cause epigenetic modifications inherited through cell divisions (32, 33). Such modifications could impact on cell fate with secondary effects on proliferation. Alternatively deregulation of cell proliferation could impact directly on differentiation and cell fate as shown recently in *Drosophila* neuroblasts (34). It is thus difficult to analyze the direct effect of *RBR* on differentiation in experimental strategies perturbing *RBR* function during a large number of cell divisions before differentiation takes place.

In contrast to organogenesis of vegetative tissues, male gametogenesis comprises only 2 cell divisions. The first asymmetrical division of the meiotic microspore produces the larger vegetative cell and the smaller generative cell, which functions as a germ cell. The germ cell divides equally only once, producing 2 identical sperm cells. The differentiated vegetative cell produces the pollen tube, which delivers the 2 sperm cells to the 2 female gametes (35). In half of the haploid *rbr* microspores from heterozygous *rbr/+* plants, the sudden deprivation of a functional *RBR* allele allows monitoring of the direct effect of the loss of *RBR* on cell proliferation and cell fate in the developing pollen.

We report that loss of *RBR* causes limited over-proliferation of the 2 pollen cell types. We further study the effect on cell fate using several markers and observe only a limited impact of *rbr*. The *rbr* phenotype is completely reversed in the absence of the cyclin dependent kinase A, leading to the hypothesis that *rbr* primarily targets cell cycle regulation with a secondary impact on cell fate.

Results and Discussion

We observed expression of *RBR* throughout pollen development in all cell types [supporting information (SI) Fig. S1]. Two mutant alleles *rbr-1* (24, 26) and *rbr-2*, show reduced paternal transmission (Table S1) linked with reduced pollen viability (Fig. S2). We further characterized at the cellular and

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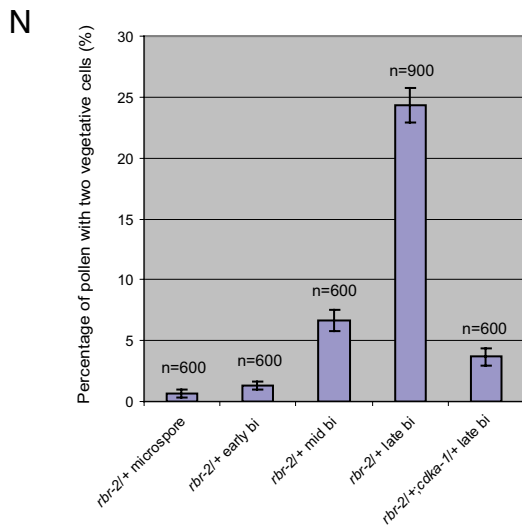
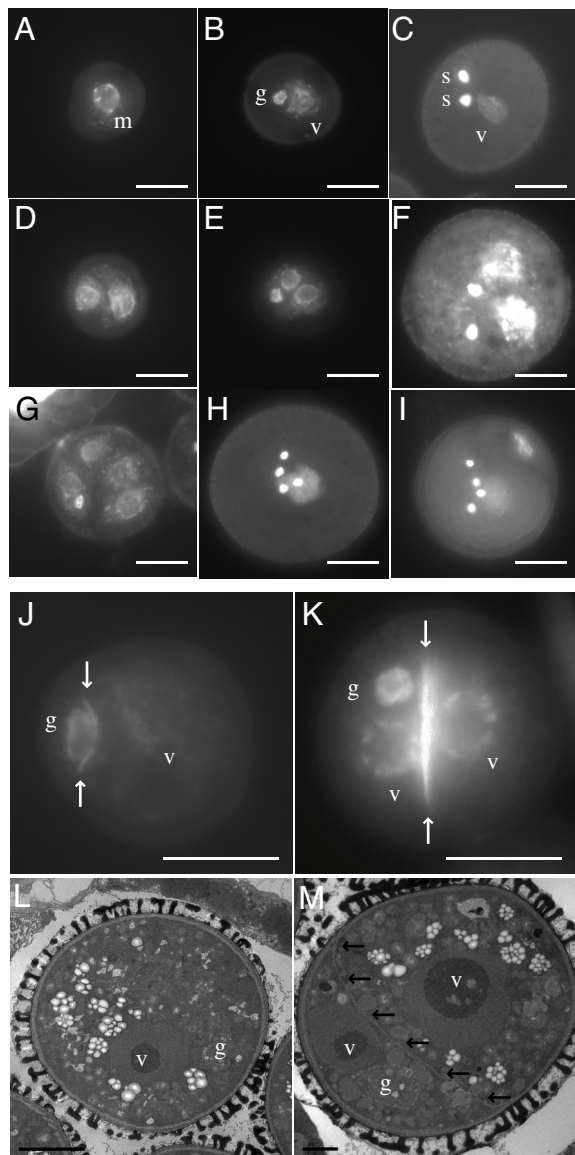


Fig. 1. Cell over-proliferation during pollen development in *rbr/+* mutants. (A–C) WT pollen development. (A) The microspore with the undetermined cell fate undergoes an asymmetrical mitosis, leading to bicellular pollen (B). At

molecular levels the defects caused by *rbr* mutations during pollen development.

Limited Cell Over-Proliferation in *rbr* Pollen. A recent study reported hyperproliferation of the vegetative nucleus of *rbr* pollen (26), but the origin of the supernumerary cells was not analyzed. We studied development of *rbr* pollen with nuclei stained by DAPI. WT microspores never divide equally ($n = 1,000$; Fig. 1A). In contrast, we observed in *rbr/+* plants a very small fraction of microspores 0.67% ($n = 600$) that had divided equally into 2 cells (Fig. 1D). The very limited impact of *rbr* on microspore division might be explained by inheritance of residual RBR from the *rbr/+* meiotic precursor.

The WT bicellular pollen comprises a vegetative cell with a large nucleus with de-condensed chromatin, and a smaller generative cell with a smaller nucleus (Fig. 1B). In contrast, *rbr/+* plants produced 24.3% ($n = 900$) pollen containing 3 nuclei (Fig. 1E). One nucleus displayed the condensed chromatin typical of generative cells. The other 2 nuclei were larger with less condensed chromatin typical of vegetative cells (Fig. 1B and E). Wild-type bicellular pollen is marked by a transient eccentric cell wall (Fig. 1J and K). In contrast, the abnormal 3-celled *rbr* pollen observed at the bicellular WT stage showed an aberrant cell wall between the 2 vegetative cells (Fig. 1K and M). The proportion of pollen containing 2 vegetative cells rose sharply during late bicellular stage, affecting half of the *rbr* pollen (Fig. 1N). We never observed any 3-celled pollen at that stage, suggesting that *rbr* causes an ectopic division of the vegetative cell.

Half of the pollen produced by *rbr/+* plants inherits the *rbr* mutation. We estimated that 30% of the *rbr* pollen was dead at the bicellular stage (Fig. S2B; percentages are expressed relative to the estimated *rbr* pollen population and are thus twice as shown on Fig. S2B). Fifty percent of *rbr* pollen showed abnormal development and 20% showed WT morphology. At the tricellular stage, at least 60% of the *rbr* pollen was dead (Fig. S2B). As *rbr-2* male transmission rate is of the order of 10% (Table S1), we could assume that 20% *rbr* pollen with normal morphology at bicellular stage underwent further development as WT. We thus estimated that, at the tricellular stage, less than 20% of the *rbr* pollen would derive from abnormal 3-celled pollen observed at bicellular stage. Corresponding to our estimate, we observed

that stage the pollen grain composes a large vegetative cell containing a small germ cell with a nucleus showing relatively higher chromatin compaction. The germ cell divides into 2 sperm cells with highly condensed chromatin, leading to the tricellular pollen grain (C). (D–I) *rbr* pollen development. Cell fates are determined on the basis of nuclear morphology. (D) At the microspore stage, *rbr* pollen grain with 2 undetermined cell nuclei. (E) Bi-cellular-stage *rbr* pollen grain with 2 vegetative cell nuclei and 1 germ cell nucleus. (F) Tri-cellular-stage *rbr* pollen with 2 vegetative cell nuclei and 2 sperm cell nuclei. (G) Tri-cellular-stage *rbr* pollen with 4 vegetative cell nuclei and 1 germ cell nucleus. (H) Tri-cellular-stage *rbr* pollen with 1 vegetative cell nucleus and 4 germ cell nuclei. (I) Tri-cellular-stage *rbr* pollen with 2 vegetative cell nuclei and 4 germ cell nuclei. Nuclei are stained with DAPI. (Scale bars, 10 μm .) (J) Bi-cellular-stage WT pollen. The cell wall (arrows) is asymmetrically placed between the vegetative nucleus and the generative nucleus. (K) Bi-cellular-stage *rbr* pollen. The cell wall (arrows) is symmetrically placed between the 2 vegetative nuclei. Nuclei are stained with DAPI, and the cell walls are stained with aniline blue. (L and M) Transmission electron micrographs of bi-cellular-stage WT pollen (L) and *rbr* pollen (M). Note the internal wall indicated by arrows in *rbr* pollen. (Scale bars, 10 μm in J and K; 5 μm in L; 2 μm in M.) (N) Bar chart showing percentage of the pollen contains 2 vegetative cells in *rbr-2/+* mutants at microspore, early bi-cellular, mid-bi-cellular, and late bi-cellular stages. At late bi-cellular stage, the over-proliferation in pollen from *rbr-2/+;cdka-1/+* plants was reduced to one seventh of the over-proliferation in pollen from *rbr-2/+* plants. Error bars correspond to SEs calculated on the basis of several samples of 100 pollen grains, and the size of total population analyzed (n) is indicated above each column. *m*, microspore nucleus; *g*, germ cell nucleus; *v*, vegetative cell nucleus; *s*, sperm cell nucleus.

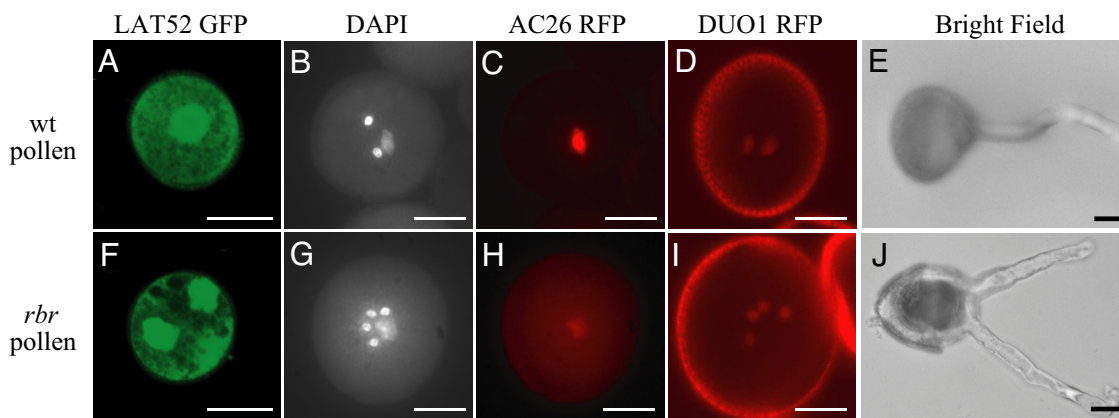


Fig. 2. Cell fate specification in *rbr* pollen. (A–E) WT pollen grains. (F–J) *rbr* pollen grains. (A and F) Bi-cellular pollen grains expressing the vegetative cell marker *pLAT52-GFP*. (B and G) Fluorescence images of tri-cellular pollen grains stained with DAPI (C and H) The same pollen grains as B and G, respectively, expressing the vegetative cell marker *pAC26-H2B-mRFP*. WT pollen grain with 1 vegetative cell and 2 sperm cells (B) has only the vegetative cell nucleus expressing *pAC26-H2B-mRFP* (C). *rbr* pollen with 1 vegetative cells and 4 germ cells (G). Only the vegetative cell nucleus expresses *pAC26-H2B-mRFP* (H). (D and I) Tri-cellular pollen grains expressing the germ cell marker *pDUO1-DUO1-mRFP*. (E and J) In vitro pollen germination. WT pollen produces only 1 pollen tube germinated (E), whereas 2 pollen tubes germinated from the same *rbr* pollen grain (J). (Scale bars, 10 μ m.)

a total of 8% of abnormal pollen grains showing a complex array of phenotypes ($n = 1000$ pollen from *rbr-2/+* plants). A predominant class of abnormal pollen contained 2 vegetative nuclei and 2 small sperm-like cells (4.6%; Fig. 1F). This class of abnormal pollen likely originated from the class shown in Fig. 1E in which either the generative cell divided into 2 sperm-like cells or the additional vegetative cell divided again, producing a generative cell. Several other types of pollen were observed (Fig. 1 G–J). Some pollen contained 4 vegetative nuclei and 1 sperm-like nucleus (1.6%; Fig. 1G). This pollen class likely results from an additional division of the 2 vegetative cells followed by 1 unequal division of 1 of the 4 vegetative cells producing a generative-like cell. We also observed pollen containing 4 sperm nuclei, either associated with 2 vegetative-like nuclei (1.2%; Fig. 1J) or inside 1 vegetative cell (3.4%; Fig. 1H). The latter class probably originates from a supernumerary division in the germ lineage. We did not observe any of the aforementioned phenotypes among WT pollen ($n > 300$ for each stage).

We targeted partial down-regulation of *RBR* in each pollen cell type by the expression of *RBR* hairpin RNAi constructs. Transgenic lines expressing the *RBR* RNAi construct under the control of the germ line-specific promoters of *HTR10* (33,36,37) (47 lines observed) and *GEX2* (38) (34 lines observed) did not show any defect in pollen viability or phenotype. In contrast, *RBR* RNAi expression restricted to the vegetative cell using the *LAT52* promoter (39) caused a distinct increase in vegetative nuclear DNA fluorescence (Fig. S3) in 10%–25% of pollen, reflecting increased DNA synthesis. However we did not observe ectopic division of the vegetative cell. Hence, *RBR* RNAi expression under the *LAT52* promoter caused a limited reduction of *RBR* activity leading to defects milder than the complete loss of *RBR* in *rbr* mutant alleles.

We conclude that *rbr* loss of function mostly affects the vegetative lineage and prevents arrest of cell division typical of vegetative cell fate. The loss of *rbr* function does not cause more than 2 additional rounds of cell division in comparison to WT. Further hyperproliferation in *rbr* pollen may be prevented by the limited supply of nutrients during pollen development leading to developmental arrest or death.

Cell Fate in *rbr* Pollen. The nuclear morphology in *rbr* pollen suggested that cell over-proliferation in *rbr* pollen grains was associated with correct vegetative and germ cell fates. To address

this question, we analyzed the expression of 6 cell fate markers in *rbr* pollen (Figs. 2 and 3). In the pollen displaying the rare phenotypic classes with duplication of the vegetative or germ cell lineages (Fig. 1I), the *rbr* pollen expressed the vegetative cell markers *pLAT52-GFP* (40) (Fig. 2F; $n = 42$) and *pAC26-H2B-mRFP1* (41) (Fig. 2H; $n = 14$) in the large vegetative-like cells and the germ line marker *pDUO1-DUO1-mRFP1* (42) in the small germ-like cells (Fig. 2I; $n = 9$). These observations suggested that *rbr* does not affect cell fate in this class of pollen. Accordingly we observed that 0.3% ($n = 1,327$) of pollen grains from *rbr* mutant germinated 2 pollen tubes likely originating from 2 vegetative cells (Fig. 2J). We concluded that, despite cell over-proliferation in *rbr* pollen, the vegetative cell fate and sperm cell fate are not affected when pollen experiences a complete duplication.

We further studied the cell fates in the 3-celled *rbr* pollen, most representative of the *rbr* phenotype at WT bicellular stage (Fig. 3). In WT bi-cellular pollen, the germ cell expresses the markers *pHTR10-HTR10-mRFP* (Fig. 3A) and *pAC24-mRFP* (41) (Fig. 3D). In two thirds of *rbr* pollen with 1 germ cell nucleus and 2 vegetative cell nuclei, the markers were correctly expressed (Fig. 3 B and E). However, in a third of 3-celled *rbr* pollen, both the germ cell nucleus and one of the vegetative-like nuclei expressed the germline markers (Fig. 3 C and F). Such ectopic expression was never observed in WT pollen ($n > 300$ for each marker). In addition, when we observed the co-expression of the vegetative marker *pLAT52-GFP* and the germline marker *pDUO1-DUO1-mRFP* (Fig. 3 G–J), a quarter of *rbr* pollen expressed the germline fate marker incorrectly. The additional vegetative-like cell expressed either the germline marker ($n = 13$ of 76; Fig. 3I) or both markers simultaneously ($n = 7$ of 76; Fig. 3J). We never observed mis-expression of the vegetative marker in the *rbr* germline ($n = 76$).

The *rbr* vegetative cell appears to behave like a microspore attempting imperfectly to reiterate an unequal division, producing an additional cell with vegetative fate, germ cell fate, or mixed fate identity. According to this hypothesis, genes expressed in the microspore but not later in the vegetative cell should be expressed in the vegetative cells of *rbr* pollen. Immunolocalization of the centromeric histone 3 variant HTR12 in WT tri-cellular pollen had shown that this protein marks only sperm cell nuclei (43). Accordingly, the centromeric histone HTR12 fused to GFP (HTR12-GFP) placed under the control

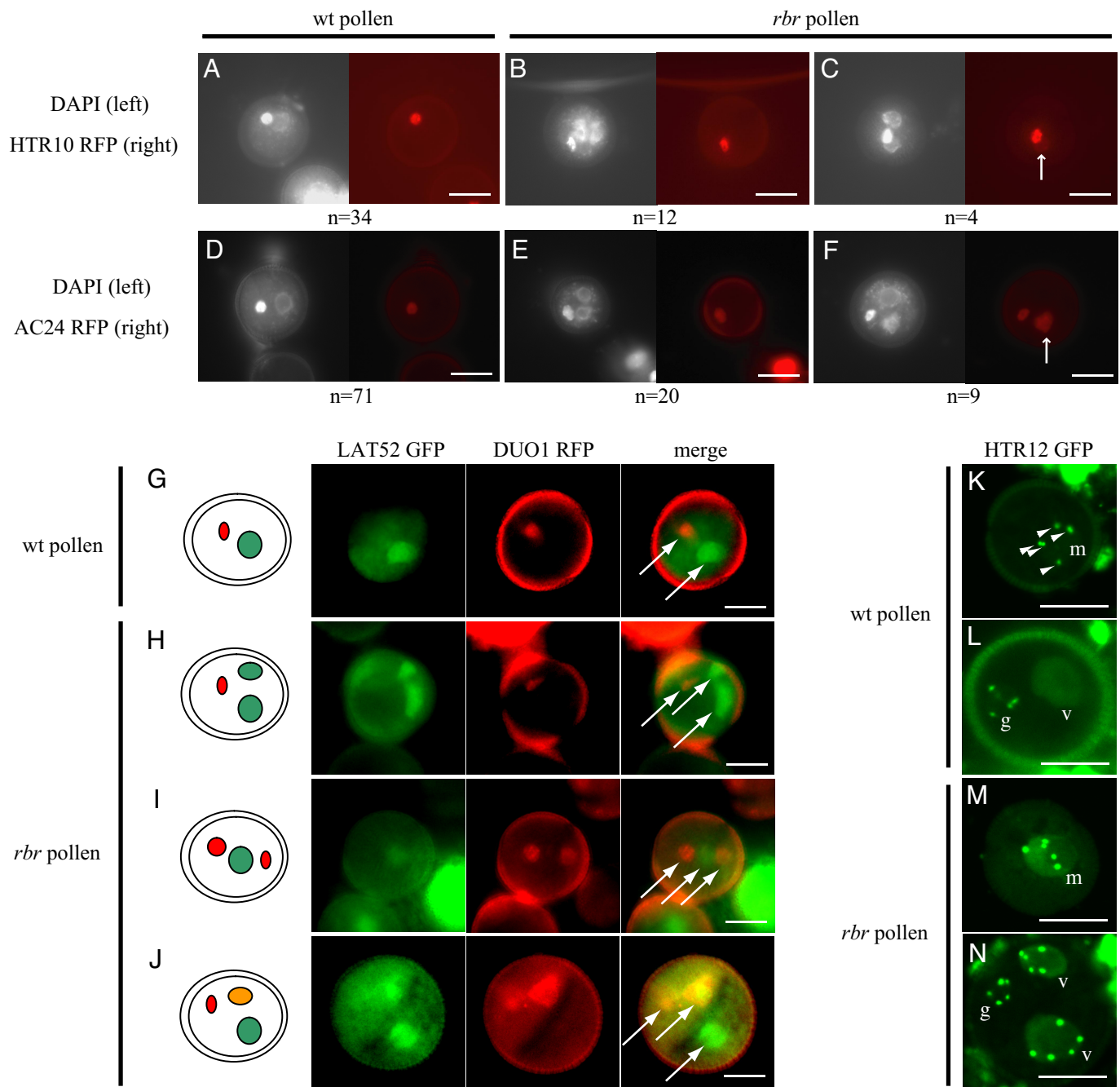


Fig. 3. Mis-specification of cell fate in *rbr* pollen. (A–C) Fluorescence images of bi-cellular-stage pollen grains stained with DAPI (Left) and expressing *pHTR10-HTR10-mRFP* (Right). (D–F) Fluorescence images of bi-cellular-stage pollen grains stained with DAPI (Left) and expressing *pAC24-H2B-mRFP* (Right). Below each figure, *n* indicates the number of each case observed. (G–I) Fluorescence images of bi-cellular-stage pollen grains co-expressing the vegetative marker *pLAT52-GFP* and the germline marker *pHTR10-HTR10-mRFP*. Panels (Left to Right) are schematic representation of pollen co-expressing the 2 markers, GFP channel, RFP channel, and merged image. Arrows indicate the positions of cell nuclei. (K–N) Fluorescence images of microspores (K and M) and bi-cellular-stage pollen grains (L and N) expressing the centromeric Histone 3 variant fused to GFP (*HTR12-GFP*). In WT (K and L), *HTR12-GFP* accumulates at the 5 chromocenters (arrowheads) of the microspore nucleus (m) (K) and the germ cell nucleus (g) (L), but it is not possible to distinguish chromocenters in the vegetative cell nucleus (v). In contrast, in *rbr* pollen, *HTR12-GFP* is detected at chromocenters in microspores (M) and in both cell types at bi-cellular stage (N). (Scale bars, 10 μ m.)

of its own promoter (44) was expressed in the WT microspore (Fig. 3K) but was no longer detected in the vegetative cell nucleus after bi-cellular stage (Fig. 3L). *HTR12-GFP* expression was observed in all microspores from *rbr-2/+* plants ($n = 100$; Fig. 3M), suggesting that RBR did not have a major impact on *HTR12-GFP* expression at that stage. In contrast to WT bicellular pollen, *rbr* 3-celled pollen showed ectopic expression of *HTR12-GFP* in vegetative cells ($n = 24$; Fig. 3N). This obser-

vation supported our hypothesis that the *rbr* vegetative cell retains the undetermined identity of the microspore. We thus concluded that *rbr* prevents cell fate establishment in the vegetative cell. A non-exclusive alternative explanation is that increased DNA methylation activity caused by increased *MET1* expression in *rbr* background (27) impacts on heterochromatin organization, causing *HTR12* recruitment. Our results thus led us to propose that loss of retinoblastoma function prevents cell

fate establishment during male gametogenesis with an impact that depends on the cell type.

***rbr* Pollen Defects Are Rescued by Dereglulation of the Cell Cycle.** Perturbation of the RBR pathway by over-expression of cyclin D3 impacts on cell proliferation and the timing of endo-reduplication in leaves and other vegetative tissues (45, 46). As endoreduplication usually marks differentiation in vegetative tissues, it was proposed that the cyclin D pathway controls cell differentiation (45). Although the impact on cell fate was not directly established in these studies, it is possible that the cyclin D pathway associated with cyclin-dependent kinase A (CDKA) regulates RBR function (47) and mediates the transition toward differentiation via the promotion of endo-reduplication in plants.

We further hypothesized that, if *rbr* directly prevents cell commitment to differentiate, preventing hyperproliferation in an *rbr* background should not rescue the defective cell fate in *rbr* pollen. To prevent cell proliferation without affecting cell fate, we choose to manipulate the Cyclin Dependent Kinase A (CDKA), which controls RBR licensing of the entry to S phase but presumably not the involvement of RBR in chromatin remodeling complexes. In animals, a few reports have shown involvement of CDKA homologues in cell fate in *Drosophila* (48) and in *C. elegans* (49). However, the mechanisms involving Cdk in cell polarity remain unclear. In *Arabidopsis* the function of the major Cdk CDKA has been solely linked to the control of the cell cycle in vegetative tissues (50) and during male gametogenesis (51–53). We thus rationalized that antagonizing RBR regulation of the cell cycle by CDKA manipulation would allow us to uncouple RBR functions in cell cycle regulation from other functions related to chromatin regulation. We tested in *rbr-2* pollen the effect of hypo-proliferation caused by the loss-of-function *cdka* mutant allele. In the *rbr-2/+;cdka-1/+* double mutant, we studied the transmission of *rbr-2* and the phenotype of the pollen. The presence of *cdka-1* almost completely rescued the paternal transmission efficiency of *rbr-2* (Table S1), in agreement with the prediction of a complete viability of the *rbr-2; cdka-1* ($z = 32.52$, $P < 0.000001$, 2-tailed test if no complementation; $z = -1.24$, $P = 0.1075$, 2-tailed test if full complementation). Accordingly, pollen lethality (Fig. S4) and over-proliferation (Fig. 1N) were greatly decreased in *rbr-2/+; cdka-1/+* plants. The percentage of defective pollen was decreased by more than half in *rbr-2/+; cdka-1/+* plants in comparison to that from *rbr-2/+* plants, both at bicellular and tricellular stages (Fig. S2B), leading to full rescue of pollen death in *rbr-2/+; cdka-1/+* plants.

It thus appears that restoring proliferation to WT levels in an *rbr* background rescues the defects in cell fate establishment observed in the *rbr* mutant. We propose that the primary effect of the loss of function of RBR in male gametogenesis is mediated by its role in cell proliferation.

Conclusions

Our study suggests that the control of the degree of proliferation by RBR is essential for proper cell fate establishment during male gametogenesis. One scenario is that the loss of retinoblastoma function primarily promotes hyperproliferation with secondary effects on commitment to cell fate during early development. It is not clear how cell fate is established in the bicellular pollen, but gradients of fate determinants have been hypothe-

sized (35, 55). The additional cells produced by the *rbr* pollen might be positioned improperly relative to developmental cues, causing anomalous or mixed-cell fate. An alternative scenario proposes that RBR directly coordinates cell division and cell fate commitment. This could be mediated directly by the cell cycle machinery as suggested by a role of CDKA homologues in cell fate reported in a few cell types (48,49,56). A third non-exclusive hypothesis relates to the role of the Rb protein in chromatin modifications. In mammals it was shown that the Rb protein interacts with several chromatin remodeling complexes (6–9,11). These complexes might be conserved in plants. Cell fate establishment would then require chromatin modifications dependent on DNA duplication. We propose that, in the absence of RBR function, hyperproliferation coupled to the absence of recruitment of chromatin modifying complexes prevents this cell fate establishment.

Experimental Procedures

Plant Strains and Growth Conditions. The WT ecotype Columbia (Col-0) was provided by the Nottingham *Arabidopsis* Stock Centre. The *A. thaliana* *rbr* mutant alleles (Columbia accession) used in this study were *rbr-2* (SALK_002946; SALK collection), and *rbr-3* (GABI170G02; GABI-Kat collection) (23). Marker lines for cell identity were *pDUO1-DUO1-mRFP* (C24) (42), *pAC24-H2B-mRFP*, *pAC26-H2B-mRFP* (C24), and *pHTR10-HTR10m-RFP* (Col) (36). *pLAT52-GFP* (Col) was a gift from Alice Cheung (Amherst, MA).

RT-PCR. Pollen at different stages of development were isolated and RNA extracted as described previously (41). Total RNA was prepared using the RNeasy mini kit (Qiagen) followed by DNase treatment (Ambion). Reverse transcription was performed by M-MuLV reverse transcriptase (New England Biolabs) with RNA ribonuclease inhibitor (Promega).

RBR Hairpin Interference Plasmid Construction and Transformation. To express hairpin dsRNA targeted to RBR transcripts specifically in the vegetative cell, 500 bp of RBR coding sequence was cloned in sense and antisense orientations into a modified Gateway expression vector *pK7LAT52RNAi* harboring the vegetative cell-specific *LAT52* promoter. A 495-bp *LAT52* promoter fragment was amplified using KOD HiFi DNA Polymerase (Novagen) with primers containing restriction sites for HindIII and XhoI. The *LAT52* promoter fragment was cloned into a Gateway RNAi destination vector *pK7gwiwgl* using the HindIII and XhoI sites to generate the *pK7LAT52hpRNAi* vector. A 500-bp RBR fragment was amplified by PCR and cloned by recombination using the Gateway cloning system according to manufacturer's instructions (Invitrogen) to generate the *pLAT52hpRBR* construct. Verified plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 and used to generate transgenic lines in *A. thaliana* ecotype Col-0 using the floral dip method. Transgenic progeny were selected for kanamycin resistance.

Microscopy and Image Processing. Alexander staining and DAPI fluorescence in pollen grains were visualized as described previously (41). Light microscopy was performed on a stereomicroscope (DM6000; Leica). Images were recorded with a monochrome digital camera (Photometrics; Roper Scientific). Fluorescence was imaged using laser scanning confocal microscopy (LSM 510 META upright; Zeiss). Figures were composed with Adobe Photoshop 7.0.1 and Illustrator 10.0.3 (Adobe Systems). Transmission electron microscopy was performed with 85-nm thin sections were prepared on a Leica Ultracut UCT ultramicrotome. Samples were observed at 120 kV under a JEM-1230 transmission electron microscope (JEOL).

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