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Report

A Dynamic Reciprocal *RBR*-PRC2 Regulatory Circuit Controls *Arabidopsis* Gametophyte Development

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Summary

Unlike animals that produce gametes upon differentiation of meiotic products, plants develop haploid male and female gametophytes that differentiate gametes such as sperm, egg and central cells, and accessory cells [1, 2]. Both gametophytes participate in double fertilization and give rise to the next sporophytic generation. Little is known about the function of cell-cycle genes in differentiation and development of gametophytes and in reproduction [1, 2]. *RETINOBLASTOMA RELATED* (*RBR*) is a plant homolog of the tumor suppressor Retinoblastoma (pRb), which is primarily known as negative regulator of the cell cycle [3]. We show that *RBR* is required for cell differentiation of male and female gametophytes in *Arabidopsis* and that loss of *RBR* perturbs expression levels of the evolutionarily ancient *Polycomb* Repressive Complex 2 (PRC2) subunits and their modifiers encoding PRC2 subunits or *DNA METHYLTRANSFERASE 1* (*MET1*) [4–6], exemplifying convergent evolution involving the *RBR*-PRC2-*MET1* regulatory pathways. In addition, RBR binds *MET1*, and maintenance of heterochromatin in central cells, a mechanism that is likely mediated by *MET1* [7, 8], is impaired in the absence of *RBR*. Surprisingly, PRC2-specific H3K27-trimethylation activity represses paternal *RBR* allele, suggesting a functional role for a dynamic and reciprocal *RBR*-PRC2 regulatory circuit in cellular differentiation and reproductive development.

Results and Discussion

Cellular Differentiation Is Defective in *rbr* Male and Female Gametophytes

In *Arabidopsis*, the male gametophyte (pollen) develops from the meiotic microspore by two consecutive mitotic divisions [1]. The first asymmetric mitosis produces one vegetative and a generative cell (binucleate stage, Figure 1A), which then forms two sperm cells after a second mitosis (3-nucleate stage, Figure 1E) [1]. We previously observed that *rbr* mutants had severely reduced transmission of the paternal *rbr* allele and that *rbr* pollen was sterile [9]. We investigated whether loss of *RBR* function affected male gametophytic cell fate and/or differentiation. In more than 40% of *rbr/RBR* pollen, a vegetative-like nucleus had aberrantly undergone an additional round of mitosis (n = 279; Figures 1B–1D), a behavior that in the wild-type is strictly restricted to the generative nucleus. As a consequence, most of the *rbr* generative-like nuclei remained undivided, failed to form sperm cells, and aborted with diffused chromatin (n = 713; Figures 1F and 1H). Therefore, correct differentiation of male gametophytic cell types requires *RBR* function.

The female gametophyte develops within ovules by three nuclear mitotic divisions of the meiotic megaspore to produce an 8-nucleate syncytium, which differentiates into a 7-cell structure consisting of two synergids, an egg cell, a binucleate central cell, and three antipodal cells (Figure 1I) [2]. After fusion of the central cell nuclei and degeneration of the antipodal cells, the embryo sac is mature and awaits fertilization (Figure 1L). We previously reported that mature *rbr* mutant embryo sacs showed aberrant nuclear proliferation at maturity [9]; however, the exact timing and associated aberrations in differentiation events were not known. Detailed analysis by light microscopy showed that in 71% of the ovules in closed wild-type flower buds prior to anthesis, the 8-nucleate embryo sac was cellularized but polar nuclei remained unfused (n = 185; stage FG5.2; Figures 1I and 1K). At the same stage of ovule development, only 47% of the *rbr/RBR* ovules had embryo sacs at stage FG5.2, and 23% of the embryo sacs showed signs of cell proliferation (n = 168; Figures 1J and 1K; for details see Figure S1 available online). Therefore, the embryo sac proliferation phenotype of *rbr* begins only after the transient cellularization, suggesting that the first three mitotic divisions of megagametogenesis proceeded normally in the absence of *RBR*.

Although we observed mature embryo sacs at stage FG7 in 95% of ovules in the wild-type emasculated pistils (n = 294), nearly half of the ovules from *rbr/RBR* plants showed nuclear proliferation in their embryo sacs (n = 402; Figures 1L–1N; for details, see Figure S2). Proliferation occurred either in all cell types (Figure 1M; Figure S2G) or was restricted to the egg apparatus (Figures S2D) or the central cell region only (Figure S2C). In rare cases, egg and central cells developed into embryo- and endosperm-like structures in the absence of fertilization, although they did not complete seed development (Figures S2E and S2F). These data suggest that all embryo sac nuclei can contribute to the *rbr* proliferation phenotype, contrary to earlier conclusions that only the central cell nuclei proliferate in *rbr* female gametophytes [10]. Analysis of a G2-M phase marker, *pCYCB1;1-GUS* (β-glucuronidase fusion), which contains a cyclin destruction box targeting the protein for degradation at the end of M phase [11, 12], showed no cyclin B1 marker expression in the mature female gametophytes that await fertilization (Figure 1O). Therefore, the mature female gametophytic cells arrested either during G1, or perhaps they arrested in G2 phase just prior to the expression of the B1;1 cyclin, as demonstrated for the male gametes [13]. Most *rbr* embryo sacs showed no *pCYCB1;1-GUS* activity at this stage (Figures 1P and 1R), and 3% of the *rbr* embryo sacs expressed

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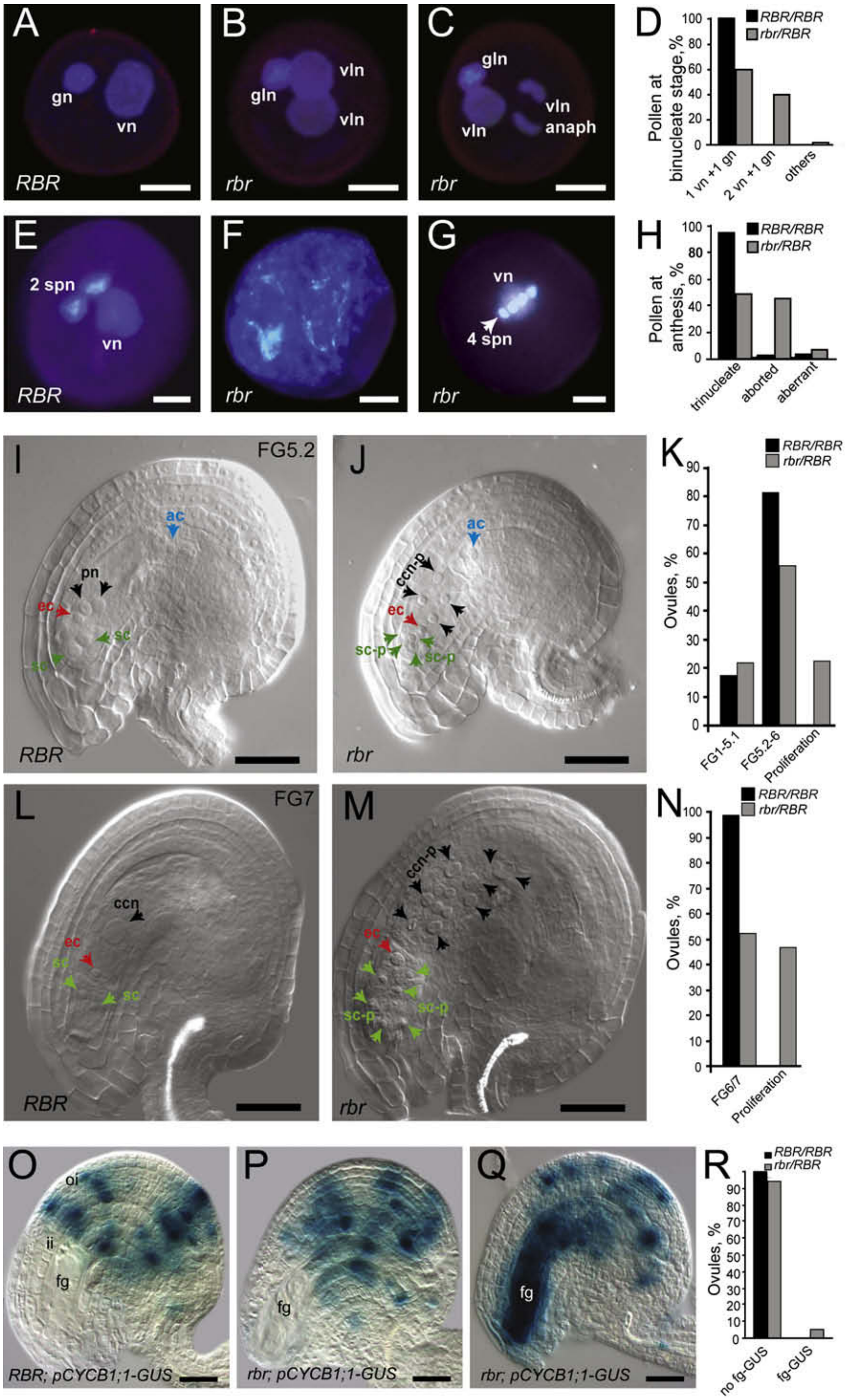

Figure 1. *RBR* Controls Cellular Differentiation of Male and Female Gametophytes

(A–H) *rbr* pollen does not differentiate the sperm cells. Shown are confocal micrographs of DAPI-stained pollen grains at binucleate stage (A–C) or mature trinucleate stage (E–G). (D, H) Histograms of the phenotypic classes. (C) One of the two *rbr* vegetative-like nuclei is at anaphase (vln anaph), suggesting a second, additional round of mitosis. (G) Rare *rbr* events such as pollen with multiple sperm cells (4 spn) were seen at maturity.

(I–N) Loss of *RBR* in the embryo sac leads to unfused polar nuclei (pn) and unrestricted cell proliferation throughout the embryo sac (ccn-p, sc-p). Shown are differential interference contrast (DIC) images of ovules with female gametophyte stage FG5-2 [40] ([I], [J], histogram in [K]) and DIC images of mature ovules 2 days after emasculation, stage FG6/7 [40] ([L], [M], histogram in [N]).

(O–R) A marker for the G2/M phase of the cell cycle, *pCYCB1;1-GUS*, was not expressed as in the wild-type in the majority of the *rbr* embryo sacs at maturity (no fg-GUS, histogram in [R]). (Q) Aberrant *pCYCB1;1-GUS* expression in the embryo sac was seen only in rare instances (fg-GUS, histogram in [R]). (R) Histogram depicting classes of ovules with distinct expression patterns.

Abbreviations: gn, generative nucleus; vn, vegetative nucleus; gln, generative-like nucleus; vln, vegetative-like nucleus; anaph, anaphase; spn, sperm nuclei; pn, polar nuclei; ccn, central cell nucleus; ec, egg cell; sc, synergid cell; ac, antipodal cells; ccn-p, proliferating central cell nuclei; sc-p, proliferating synergids; ii, inner integuments; oi, outer integuments; fg, female gametophyte.

Scale bars in (A)–(C), (E)–(G) represent 5 μm; in (I), (J), (L), (M), (O)–(Q), scale bars represent 30 μm.

this marker in some or all cells (Figures 1Q and 1R). These results suggest that *rbr* embryo sacs do not arrest cell-cycle progression, which is consistent with their nuclear proliferation phenotype. In addition, most proliferating *rbr* female gametophytes failed to express cell-specific markers that are detected in a fully differentiated wild-type mature embryo sac (data not shown), confirming that *RBR* is required for a complete differentiation of all gametophytic cells.

Although cell-cycle regulation in plant development is well understood [14], it remains unclear how cell-cycle genes function in mitotic divisions and differentiation events during male and female gametophytic development. This is because (1) gametophytes are inaccessible for large-scale studies; (2) gametophytic mutations can not be maintained in their homozygous state, and the heterozygous mutant plants produce both mutant and wild-type gametophytes in an equal ratio; and (3) cell-cycle genes in plants are represented by duplicated gene families, making the genetic analysis more difficult. In *Arabidopsis*, only a limited number of cell-cycle genes have been implicated in gametophyte development and/or subsequent embryo or endosperm development, including DNA replication licensing factors such as *PROLIFERA* [15], *REPLICATION FACTOR C* (*RFC1/3*) (http://www.seedgenes.org), and *ORIGIN REPLICATION COMPLEX 2* (*ORC2*) [16], chromosome scaffold proteins of the condensin (SMC2) and cohesin (SMC1/3) classes (http://www.seedgenes.org, [17]), genes of the anaphase-promoting complex or cyclosome (*APC1/2*, *NOMEGA*) that regulate mitotic progression (http://www.seedgenes.org, [18, 19]), and *CDKA;1*, a cdc2 homolog [20, 21]. *RBR* is the first cell-cycle gene in plants, however, that is essential during development of the gametophytes and the sporophyte [9, 22]. Our current work further suggests that in both male and female gametophytes, *RBR* connects cell-cycle control to cellular differentiation processes.