Retinoblastoma and Its Binding Partner MSI1 Control Imprinting in Arabidopsis

Pauline E. Jullien1,2, Assaf Mosquna3, Mathieu Ingouff1, Tadashi Sakata1, Nir Ohad2, Frédéric Berger1*

1 Chromatin and Reproduction Group, Temasek Life Sciences Laboratory, National University of Singapore, Singapore, Republic of Singapore, 2 Zentrum für Molekularbiologie der Pflanzen (ZMBP), Entwicklungsgenetik, Universität Tübingen, Tübingen, Germany, 3 Department of Plant Sciences, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel

Parental genomic imprinting causes preferential expression of one of the two parental alleles. In mammals, differential sex-dependent deposition of silencing DNA methylation marks during gametogenesis initiates a new cycle of imprinting. Parental genomic imprinting has been detected in plants and relies on DNA methylation by the methyltransferase MET1. However, in contrast to mammals, plant imprints are created by differential removal of silencing marks during gametogenesis. In Arabidopsis, DNA demethylation is mediated by the DNA glycosylase DEMETER (DME) causing activation of imprinted genes at the end of female gametogenesis. On the basis of genetic interactions, we show that in addition to DME, the plant homologs of the human Retinoblastoma (Rb) and its binding partner Rab48 are required for the activation of the imprinted genes FIS2 and FWA. This Rb-dependent activation is mediated by direct transcriptional repression of MET1 during female gametogenesis. We have thus identified a new mechanism required for imprinting establishment, outlining a new role for the Retinoblastoma pathway, which may be conserved in mammals.

Introduction

Genomic imprinting causes parental allele-specific expression during early development in mammals and plants [1]. In mammals, silencing marks are acquired by de novo DNA methylation during gametogenesis. Each imprinted locus receives a mark depending on its parental origin [2]. In embryonic cells, epigenetic marks are maintained on the silenced allele by a semiconservative mechanism involving the DNA methyltransferase Dmnt1 [3]. Following DNA replication, Dmnt1 methylates preferentially hemimethylated DNA, resulting in the maintenance of silencing marks on one parental allele. In the embryo, imprinting marks are read, leading to monoallelic gene expression. Certain genes remain imprinted and expressed in the adult [4]. A new imprinting cycle is initiated in the embryonic primordial germ cells where the epigenetic marks are lost following a global demethylation of DNA. The mechanism by which such demethylation originates is still unknown [5].

Embryogenesis in flowering plants and mammals depends on the function of embryo-nurturing annexes, the endosperm in plants and the placenta in mammals. In mammals, the placenta derives from the trophoblastic lineage separated from the embryonic lineage at the blastocyst stage. In flowering plants, the endosperm lineage separates from the embryonic lineage during female gametogenesis before fertilization [6]. The meiotic spore produces the haploid gametophyte in which two specialized female gametes differentiate: the egg cell and the central cell [7]. The fertilized egg cell produces the embryo, and the fertilized central cell generates the endosperm [8]. All imprinted genes known to date in plants are solely expressed in the endosperm, and some of these are essential for endosperm development [9].

Only a few imprinted genes have been identified in Arabidopsis [1]. MEDEA (MEA) [10], FWA [11], and FERTILIZATION INDEPENDENT SEED 2 (FIS2) [12] are only expressed from the maternal allele, whereas PHERES1 (PHE1) is preferentially expressed from the paternal allele [13, 14]. PHE1 and FWA encode transcription factors of unknown function in the endosperm. MEA and FIS2 encode subunits of a Polycomb Group (Pc-G) complex [15]. Both alleles of MEA, FIS2, and FWA are silenced by distinct epigenetic mechanisms throughout the plant lifecycle until gametogenesis takes place. Silencing of MEA results from histone 3 lysine 27 (H3K27) trimethylation by Pc-G complexes [16, 17], whereas silencing of FWA and FIS2 is mediated by the DNA METHYLTRANSFERASE 1 (MET1), which maintains DNA methylation of CpG sites [11, 12]. Silencing marks are maintained in the sperm cells during male gametogenesis. During endosperm development, the inherited paternal copy remains silenced by MET1 or Pc-G activities, whereas the maternal copy is inherited as transcriptionally active, which results in monoparental expression [1].

During female gametogenesis, the expression of MEA, FIS2,
Imprinting in plants and mammals involves a process whereby one of the two inherited gene variants (alleles) is inactivated. During imprinting, the transcriptional silencing of one allele is mediated by histone modifications or DNA methylation. The expressed parental allele is activated during gametogenesis by poorly understood mechanisms that remove silencing marks. In Arabidopsis, we studied genes expressed only from the maternal allele because the paternal allele is silenced by DNA methylation. We report that the expression of the maternal allele requires the repression of transcription of the major DNA methyltransferase by the sustained activity of the Arabidopsis homologs of the Retinoblastoma pathway. Repression is confined to the female gamete and is essential for the expression of imprinted genes in plants. The conserved transcriptional repression of DNA methyltransferases by the Retinoblastoma pathway suggests that this new regulation of imprinting might be also active in mammals.

and FWA is activated specifically in the central cell [18,19]. The activation of the above genes relies on DEMETER (DME), a DNA glycosylase protein [11,12,19]. DME removes methylated cytosines [16,20], causing the loss of the silencing marks from FIS2 and FWA promoters and allowing transcription in the central cell. The maternal allele inherited from the central cell remains active after fertilization in the endosperm while the paternal allele remains silenced. Hence the gene is imprinted in the endosperm while silenced in all other tissues. Loss of DNA methylation from the promoter of imprinted genes is conserved in the maize central cell [21,22]. Imprinting in plants thus results from the loss of silencing epigenetic marks during gametogenesis, which leads to gene expression. However, persisting expression of FIS2 in the dme mutant [12] suggested that mechanisms parallel to DME lead to removal of DNA methylation marks from FIS2 during female gametogenesis.

In mammalian cell cultures, the expression of the DNA methyltransferase Dnmt1 is controlled by the Retinoblastoma pathway [23–25]. The Retinoblastoma protein (pRb) is known to repress the expression of S-phase genes during the G1 phase of the cell cycle through inhibition of E2F transcription factors. The pRb binding protein RbAp48 is critical for this function [26]. The Arabidopsis pRb homolog RETINOBLASTOMA RELATED 1 (RBR1) is highly expressed in the mature central cell, preventing uncontrolled syncytial proliferation [27]. MULTICOPYSUPPRESSOR OF IRA1 (MSI1) protein, a RbAp48 homolog, prevents production of endosperm from unfertilized central cells [28,29]. However, the interaction between RBR1 and MSI1 was not shown in Arabidopsis. We hypothesized that in Arabidopsis, the Retinoblastoma pathway may control the expression of MET1. In this study, we demonstrate that Arabidopsis MSI1 and RBR1 interact in vivo and down-regulate MET1 directly during female gametogenesis. Reduction of MET1 activity by the Retinoblastoma pathway in the central cell is essential for transcriptional activation of FIS2 and FWA. We thus provide evidence for a new mechanism essential for the activation of MET1-dependent imprinted genes in plants.

Results

RBR1 Interacts with MSI1

Interaction between the tomato homolog of MSI1 and human or maize homologs of RBR1 has been shown in vitro [30]. Employing an in vitro glutathione S-transferase (GST) pull-down assay, we have detected direct interaction between Arabidopsis MSI1 and RBR1 proteins (Figure 1A). In order to test whether the Arabidopsis MSI1 and RBR1 proteins interact in living cells, we have used the bimolecular fluorescence complementation (BiFC) assay [31]. MSI1 and RBR1 coding sequences were fused to the sequences encoding the N-terminal (YN) or the C-terminal (YC) part of the yellow fluorescent protein (YFP). We observed YFP fluorescence from nuclei in which YN-MSI1 and YC-RBR1 were transiently cotransformed into leaf epidermis and reconstituted a functional YFP protein (Figure 1B). No fluorescence was observed using YN alone with YC-RBR1 or using YN-MSI1 with YC alone (Figure 1F and 1G). We thus concluded that MSI1 and RBR1 interact in vitro and in vivo as shown previously between homologs of RBR1 and MSI1 in human, Drosophila, and maize [32].

In order to identify which part of the RBR1 protein is required for the interaction with MSI1, we performed a deletion analysis. The Arabidopsis RBR1 protein contains the two conserved domains RB-A and RB-B (Figure 1H). Human Retinoblastoma RB-A and RB-B domains form a tridimensional structure called the A/B pocket. Human pRb interacts through the A/B pocket with the LxCxE domain of the histone deacetylase 1 (HDAC1) [33], which in turns binds RbAp48 [26,34]. Using truncations of the RBR1 protein fused to YC, we demonstrated that the interaction between MSI1 and RBR1 occurs through the RB-A domain (Figure 1B–1E). We concluded that the interaction between MSI1 and RBR1 takes place in absence of the A/B pocket, suggesting that an HDAC1 is not involved in this interaction. In addition, the maize HDAC ZmRpd3l [35] and all Arabidopsis HDAC homologs do not contain the LxCxE domain, which is required for the interaction between Rb, HDAC1, and RbAp48 in mammals. In conclusion, we suggest that in contrast to mammals, the plant homologs of Rb and RbAp48 likely interact directly and may not require an HDAC1 homolog.

MSI1 Represses the Expression of the DNA Methyltransferase MET1

To investigate the effect of MSI1 on MET1 expression, we used transgenic plants with a reduced level of MSI1 protein (MSI1cs) [36] since plants homozygote for the null msI alleles cannot be obtained due to embryo lethality [28]. Quantitative PCR (Q-PCR) analyses showed a 5-fold increase of MET1 expression in MSI1cs leaves in comparison to wild-type leaves (Figure 2A). We concluded that MSI1 represses MET1 expression. We performed bisulfite sequencing of the known methylated regions of the FIS2 and FWA promoters to determine the level of CpG methylation in plants with reduced level of MSI1. Although most CpG sites in the two regions investigated are already methylated in the wild type, we could detect a modest increase of DNA methylation in leaves of MSI1cs plants (Figure S1).

In addition to RBR1, MSI1 participates in the Pc-G complex comprising MEA, FIS2, and FIE [28,29,37]. MSI1 is also an essential component of the Arabidopsis chromatin assembly factor-1 (CAF-1) complex with the two other proteins FASCIA1 (FAS1) and FASCIA2 (FAS2) [38]. The CAF1 complex is conserved in yeast, Drosophila, and mammals and is essential for the deposition of the heterodimer H3-H4 at the replication fork [39].
In order to investigate which **MSI1**-dependent pathway is responsible for the transcriptional control of **MET1**, we tested whether **MET1** expression was affected by loss of function of essential members of three distinct **MSI1**-dependent pathways involving **RBR1**, **FIE**, or **FAS1** [32]. We failed to obtain plantlets with a significant reduction of **RBR1** expression from **RBR1** RNA interference (RNAi) lines and thus have been unable to investigate the function of the Retinoblastoma pathway (unpublished data). Reduction of **FAS1** activity in plants homozygote for the null **fas1–1** allele [38] did not modify the level of **MET1** expression (Figure 2B). Similarly, the level of **MET1** expression was not affected by reduction of **FIE** activity in **FIE** cosuppressed plants (**FIEcs**) [40] (Figure 2B). We thus concluded that **MSI1**-mediated transcriptional repression of **MET1** is independent of the Pc-G and CAF-1 pathways. We thus hypothesized that **MET1**

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**Figure 1. RBR1 Interacts with MSI1**

(A) Pull-down assay testing for interaction between *Arabidopsis* **MSI1** and **RBR1**. The full-length **RBR1** protein, labeled with [*35*S] methionine, was incubated with GST-**MSI1** protein bound to agarose beads (GST **MSI**). As a negative control, the labeled protein was incubated with GST only, bound to agarose beads (GST). Input indicates the labeled protein used in the binding assays. Immunodetection of GST and GST-**MSI1** were performed using anti-GST antibody.

(B–H) BiFC analyses showing in vivo interaction between **RBR1** and **MSI1** proteins. Fluorescence is observed in nuclei following YFP reconstitution between **YN-MSI1** and **YC-RBR1** (B). The inset represents the detail of an individual nucleus. In comparison, either **YN** with **YC-RBR1** (F) or **YN-MSI1** with **YC** (G) serving as negative controls display no fluorescence. (C–E) Truncation of the **RBR1** protein showing that only the **RB-A** domain is required for the interaction with **MSI1**. Localization was determined in leaf epidermis of *N. benthamiana*. YFP fluorescence from single confocal sections showing a fraction of the nuclei from all cells in the field was overlaid with Nomarsky differential interference contrast (DIC) images. Arrows point to nuclei expressing YFP fluorescence. Scale bars represent 20 μm. (H) Representation of the different **RBR1** truncations tested for interaction with **MSI1** in the assays shown in (A) to (G).

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**Figure 2. MSI1 Represses MET1 Expression**

(A) Q-PCR analyses on RNAs from mature leaves show an increase of **MET1** expression in **MSI1cs** in comparison to wild-type Columbia. The RQ value corresponds to the average of five independent biological replicates.

(B) Q-PCR analyses on RNAs from **FIEcs** and **fas1** mature leaves.

(C) Q-PCR analyses showing an increase of **MET1** expression in **rbr1–1/+**, **msi1–1/+**, and **fie/+** ovules at 1.5 d after emasculation (DAE) in comparison to wild-type Col (B and C). The RQ value corresponds to the average of three independent biological replicates. (A–C) Error bars represent the standard error between the biological replicates. The RQ value is represented on the top of each bar. **ACT11** was used as endogenous control for (A) and (B), **GAPC** for (C).

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transcriptional control by MSII may involve the Retinoblastoma pathway.

MSII and RBR1 Bind to the MET1 Promoter

To test whether MSII and RBR1 control directly MET1 transcription by binding to the MET1 promoter, we performed chromatin immunoprecipitation (ChIP) using antibodies against MSII (Figure S2) and RBR1 [41]. We focused our analysis on a 992-bp region of the MET1 promoter spanning −869 bp to +123 bp relative to the predicted translation start site (Figure 3A). Chromatin from wild-type Columbia buds was immunoprecipitated with antibodies against MSII or RBR1. A significant enrichment in MSII and RBR1 binding was observed for the DNA fragment 2 spanning −608 bp to −303 bp relative to the start codon (Figure 3B and 3C). We conclude that MSII and RBR1 bind to the MET1 genomic locus on a domain containing a putative E2F binding site (Figure 3A). These results strongly support the concerted action of MSII and RBR1 in regulating MET1 expression.

MSII and RBR1 Repress MET1 Expression during Female Gametogenesis

In plants with the genomic reporter construct pMSII:MSII-mRFP, we observed that MSII-RFP was expressed throughout female gametogenesis (Figure 4) similar to RBR1 expression [27]. Coexpression of MSII and RBR1 during female gametogenesis suggested that MET1 expression is down-regulated in a specific manner in the female gametes. We obtained transgenic plants expressing the HISTONE2B fused to red fluorescent protein (RFP) under the control of the MET1 promoter (pMET1-H2B-RFP). We determined the pattern of MET1 expression during female gametogenesis by confocal microscopic observations of developing ovules from four independent transgenic lines. The H2B-RFP signal was observed in nuclei of the ovule integuments and in nuclei of the syncytial embryo sac from the megaspore till the four-nuclei stage (Figure 5A–5C). At the eight-nuclei stage, the signal can no longer be detected in the embryo sac apart from the three antipodal nuclei (Figure 5D). After cellularization of the eight nuclei, we could not observe any signal in the central cell, egg cell, and synergids, although some weak signal persisted in the three antipodals (Figure 5E). In the mature gametophyte, we could no longer detect any fluorescence from H2B-RFP in the central cell marked by pFWA-GFP expression (Figure 5F). As the result of our observations, we concluded that MET1 is transcriptionally repressed in the central cell. Taking into account the expected H2B-RFP retention in nuclei after nuclear division, we estimated that MET1 activity is down-regulated at least from the four-nuclei stage of the female gametophyte.

In order to test the action of MSII on MET1 during female gametogenesis, we introduced the MET1 transcriptional reporter in the msii mutant background. In msii−/− plants hemizygous for pMET1-H2B-RFP, the RFP signal was detected in about 25% of female gametophytes, the expected proportion of ovules inheriting both msii− and pMET1-MET1-RFP (Figure 6C and 6D). This result suggested the down-regulation of MET1 expression by MSII in female gametophytes. We also observed an increased level of MET1 transcripts in ovules from msii+/+ plants (Figure 2C), confirming that MSII inhibits MET1 expression in the female gametophyte. We did not detect any change of pMET1 activity in the female gametophyte of the fie mutant (Figure 6D), showing that MET1 down-regulation in the female gametophyte is independent of FIE. However, we did observe an increased level of MET1 transcripts in ovules from fie+/+ plants (Figure 2C). Increased cell division activity in fie ovule integuments [27] could be responsible for the global increased MET1 expression linked to its cell cycle dependence. To test whether RBR1 was also required for the down-regulation of MET1 in the female gametophyte, we studied pMET1-H2B-RFP expression in rbr1 mutant. We observed ectopic pMET1-H2B-RFP expression in ovules from rbr1−/− plants (Figure 6B). RFP signal was observed in about 25% of female gametophytes from rbr1+/+ plants hemizygous for the pMET1-H2B-RFP reporter (Figure 6D). We also directly observed increased MET1 expression in rbr1+/+ ovules (Figure A B C

Figure 3. MSII and RBR1 Bind to the MET1 Promoter
(A) Schematic diagram of the MET1 locus representing the fragments (black rectangles) analyzed by PCR after ChIP. White boxes represent the 5′ UTR, and the gray arrow corresponds to the first amino acid of exon 1 of MET1. A putative E2F binding site is represented by a black dot.
(B) ChIP analysis using antibodies specific for MSII (MSII Ab) and RBR1 (RBR1 Ab) proteins. Nuclear extracts were prepared from wild-type Columbia buds after cross-linking. The first lane represents the input DNA. Control IgG is used as a negative control, while an antibody against histone 3 (H3) is used as a positive control.
(C) Absolute quantification of the ChIP using Q-PCR for the fragment 2. Error bars represent the standard deviation of two independent PCR reactions.
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after fertilization (Figure 7A). Accordingly, we would expect 25% of ovules with the inactive maternal allele of MS11 to be fertile. This result indicates that the inactivated status of MS11 is necessary for FIS2 expression in the central cell and that the loss of MS11 in the central cell prevents the expression of FIS2 in the endosperm.

To test whether the two MS11-containing complexes, Pc-G and CAF1, are responsible for the repression of FIS2 expression in absence of MS11, we studied the effect of mutants in the genes MEA, FIE, and FAS1. We did not observe any loss of FIS2-GUS expression in ovules nor in endosperm of mea-6/+; fie-11/+; and fas1-1/+ mutants (Figure S3). Thus, activation of FIS2 expression by MS11 is independent from the Pc-G and CAF-1 activities.

Altogether, the in vivo interaction between RBR1 and MS11, their coexpression in the central cell, and their concerted regulation of MET1 transcription, suggested a common requirement of MS11 and RBR1 in the regulation of FIS2 expression. Plants heterozygous for rbr1-1/+ loss-of-function allele showed a 50% reduction of ovules expressing the FIS2-GUS reporter in comparison to the wild-type background (Figure 7E and 7F). This reduction was correlated with the inheritance of rbr1 in half of the ovules ($\chi^2 = 0.27$, $p > 0.6$). A dramatic reduction of FIS2 expression was also detected by RT-PCR in rbr1-1 mutant ovules (Figure 7G).

We thus concluded that RBR1 is necessary for FIS2 expression in the central cell.

Since FIS2 expression also depends on DME [12], we tested whether the loss of FIS2 expression could be attributed to the loss of DME expression in response to the MS11/RBR1 pathway. DME and MS11 were still expressed in rbr1-1 ovules, and conversely, the expression of MS11 and RBR1 was not reduced in dme mutant buds (Figure S4). Our results suggest that the transcriptional controls of DME and MS11/RBR1 are mutually independent from each other.

**RBR1 and MS11 Are Required for FWA Expression**

The expression pattern of FIS2 is similar to the expression patterns of the other MET1-dependent, maternally expressed imprinted gene, FWA [11,12]. We tested the effect of rbr1 and ms1 mutations on the expression of FWA using the tran-
scripional reporter FWA-GFP [11]. Only half of the ovules from msi1–2/+; FWA-GFP/FWA-GFP and from rbr1–1/+; FWA-GFP/FWA-GFP plants showed GFP expression in comparison to the expression of FWA-GFP in all wild-type ovules (Figure 8A–8D). The proportions of ovules that expressed FWA-GFP were in agreement with a gametophytic reduction of FWA-GFP expression by msi1 ($\chi^2 = 1.08, p > 0.3$) and by rbr1 ($\chi^2 = 8.86, p < 0.003$) in the mutant central cell. The repressed state of FWA-GFP also persisted in the endosperm of fertilized seeds from msi1–2/+; FWA-GFP/FWA-GFP plants (Figure S6).

Similar results were obtained using the msi1–1 allele (Figure S5). Hence, both MSI1 and RBR1 are required for FWA and FIS2 expression.

Activation of FIS2 and FWA by MSI1 Is Mediated by the DNA Methyltransferase MET1

In order to investigate the genetic relationship between the activities of MET1 and MSI1, we constructed a double-mutant plant of msi1+/+; met1+/+ carrying either FIS2-GUS or FWA-GFP reporters. In this experiment, we have used different msi1 null

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**Figure 5.** Expression the pMET1-H2B-RFP Reporter during Female Gametogenesis
Confocal images from transgenic plants expressing the pMET1-H2B-RFP construct.
(A) pMET1-H2B-RFP is expressed in the functional megaspore (fm) as well as in the ovule integument (oi). (B) Two-nucleate–stage (FG2) ovule. (C) Four-nucleate–stage (FG4) ovule. (D) Eight-nucleate–stage (FG8) ovule; pMET1-H2B-RFP expression is restricted to the antipodal nuclei (an). (E) Mature female gametophyte. (F) Colocalization of pMET1-H2B-RFP with FWA-GFP showing an absence of RFP in the mature central cell (cc), 1DAE.

ec, egg cell; sy, synergids. Stages of female gametophyte development are indicated according to Christensen et al. [68]. Arrowheads point to nuclei. Scale bars represent 10 $\mu$m.
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**Figure 6.** Control of MET1 Expression by MSI1 and RBR1
(A) pMET1-H2B-RFP in wild-type mature ovules, the expression is restricted to the antipodal cells (an) and ovule integuments (oi). (B) pMET1-H2B-RFP in rbr1–1 ovules, ectopic RFP expression is observed in the central cell (cc) and egg cell (ec). Scale bar represent 10 $\mu$m. (C) pMET1-H2B-RFP in msi1–1 ovule, ectopic RFP expression is observed in the central cell (cc) and egg cell (ec). Scale bar represent 10 $\mu$m. (D) Percentage of ovules expressing pMET1/H2B-RFP in the central cell in wild type (WT), rbr1–1/+; msi1–1/+, and fie–/+ plants. Arrowheads point to nuclei. Error bars represent the standard deviation. The n number is represented on the top of each bar.
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alleles (described in Materials and Methods). MSI1 and MET1 are located only 3.6 Mb apart from each other on chromosome 5, which is estimated as 18 cM of linkage units. Considering the recombination frequency between the two genes, we expected that msi1–/+; met1–/+ plants would produce 41% of ovules carrying both the msi1 mutation and the met1 null mutation. If the effect of msi1 and rbr1 mutations on FIS2 and FWA expression was mediated by a repression of MET1, we would have expected to observe 45.5% of ovules expressing FIS2 or FWA in msi1–/+; met1–/+ plants (Table S1).

We observed that 49.9% of the ovules of msi1–/+; met1–3/+; FIS2-GUS+ plants expressed the FIS2-GUS marker in comparison to 25.5% in the msi1–/+; FIS2-GUS+ background (Figure 9A). Thus the inheritance of met1 mutation rescues the repression of FIS2-GUS expression by msi1 ($\chi^2=4.5, p>0.03$). We observed that 37.6% of the ovules of msi1–/+; met1–/+; FWA-GFP+ plants expressed FWA-GFP in comparison to 22% in the msi1–/+ background (Figure 9B). This indicated that the met1 mutation antagonized the transcriptional repression of FWA-GFP by msi1 ($\chi^2=9.77, p>0.01$). Our results suggest that

Figure 7. Control of FIS2 Expression by MSI1 and RBR1

(A) Percentage of ovules/seeds expressing GUS in plants homozygous (HO) for FIS2-GUS in wild-type (WT) and msi1–2/+ backgrounds. The percentage of ovules/seeds is represented before pollination (BP) and 3 d after pollination (3DAP). Error bars represent the standard deviation. The n number is represented on the top of each bar.

(B and C) Photography illustrating FIS2-GUS expression in the central cell of wild-type (B) and msi1–2/+ (C) ovules. Before pollination, it is not possible to distinguish on morphological bases the gametes carrying the wild-type or the msi1–2 allele. Scale bars represent 50 µm.

(D) RT-PCR on RNAs from seeds that inherited msi1–2 maternally selected on the basis of the overexpression of the fluorescent marker KS117 [28] (5 DAP). GAPDH is used as a control.

(E) Percentage of ovules expressing GUS in plants hemizygous (HE) for FIS2-GUS in wild-type and rbr1–1/+ backgrounds. Error bars represent the standard deviation. The n number is represented on the top of each bar.

(F) FIS2-GUS expression in the central cell of rbr1–1/+ ovules before pollination. Before pollination, it is not possible to distinguish on morphological bases the gametes carrying the wild-type or the rbr1–1 allele. Scale bars represent 50 µm.

(G) RT-PCR on RNAs from rbr1–1–selected ovules showing no FIS2 expression in comparison to wild-type ovules. Selection of rbr1–1 ovules was based on the lack of fertilization and seed development at 3 DAP in contrast to the wild-type ovules that were fertilized. GAPDH is used as a control.

Retinoblastoma Controls Imprinting

![Image](image-url)
MSII and RBR1 antagonize the repressive action of MET1, which regulates the expression of FIS2 and FWA. We conclude that MET1 acts downstream of the RBR1/MSII pathway, which is in agreement with our demonstration of the repression of MET1 transcription in the central cell by the RBR1/MSII pathway.

RBR1 and MSII Are Not Required for MEA Expression

Silencing of MEA depends on H3K27 tri-methylation by Pc-G complexes [16,17], whereas silencing of FIS2 and FWA is caused by DNA methylation by the maintenance methyltransferase MET1 [12]. However, MEA activation in the central cell depends on DME action antagonized by MET1 [42]. Thus it remained to be tested whether MET1 repression by RBR1 and MSII could have a direct impact on MEA expression in the central cell. In contrast to the transcriptional repression of FIS2 and FWA mediated by msi1 and rbr1 mutants, we could not detect any impact of msi1–2 or rbr1–1 on the expression of the MEA-GUS reporter (Figures 8A, 8E–8G, and S6). Similar results were obtained using the msi1–1 allele and the reporter MEA-YFP that encodes a fusion protein that complements mea (Figure S5). We thus concluded that MEA expression in the central cell is not regulated by MSII and RBR1.

Discussion

Conservation of the Transcriptional Repression of MET1 by the Retinoblastoma Pathway

We identify a site in the putative promoter of MET1 where MSII and RBR1 associate. In addition, we show that MET1 expression is repressed by MSII. This repression does not depend on the MSII association to the complexes Pc-G and CAF1, but depends on RBR1. Our results thus support a repression of MET1 transcription by the MSII/RBR1 complex. We have found one putative E2F binding site (ATTGCAGC) situated −387 bp from the predicted translation start site of the MET1 promoter. Accordingly, MET1 expression is strongly increased in plants overexpressing the Arabidopsis E2Fa and DPa proteins, suggesting that MET1 is an E2F target gene [43,44] and MET1 is expressed during the S phase of the cell cycle [45,46]. We thus propose that the activation of MET1 transcription during the S phase requires the release of the sequestration of E2F by RBR1 at the G1/S cell cycle checkpoint. The RBR1/MSII complex would prevent E2F from activating the transcription of MET1. In human cells, the transcription of the human homolog of MET1, Dnmt1, is also repressed by the Rb/E2F pathway [24,25]. Hence maintenance of DNA methylation would be coordinated with the S phase by an E2F control, and this mechanism is likely conserved in Eukaryotes.

Repression of MET1 Is Essential for Activation of Imprinted Genes during Female Gametogenesis

MSII and RBR1 are expressed throughout female gametogenesis, and close inspection of MET1 expression during female gametogenesis showed that MET1 is specifically repressed during female gametogenesis by the Retinoblastoma pathway. The reduced expression of MET1 in female gametes could explain why the inheritance of met1 by the female gamete has no effect on endosperm and seed development [47]. In contrast, MET1 is expressed in male gametes (Figure S7), and the inheritance of met1 by the male gametes causes a strong reduction of endosperm and seed size [47,48] presumably as a result of the ectopic activation of the paternal allele of imprinted genes regulated like FWA and FIS2.

Our results indicate that the RBR1/MSII pathway activates the maternal expression of FIS2 and FWA via the transcriptional repression of MET1 in the central cell. The imprinted genes FIS2 and FWA are repressed throughout the vegetative stage by MET1-dependent DNA methylation on their promoters [11,12]. At the end of female gametogenesis, DNA methylation is removed in the central cell by DME [11,16,19] leading to transcriptional activation. The demethylated maternal allele remains active in the endosperm. DME has a high affinity for hemimethylated DNA and causes excision of methylated cytosine residues followed by repair of single-strand break and incorporation of a nonmethylated
The top of each bar. Bars represent the standard deviation. The msi1–2/FIS2-GUS hemizygous (HE) for Percentage of ovules expressing GFP or GUS activity from plants are sufficient to maintain isolated from each other. In the egg cell, the remaining marks syncytial division, the central cell and the egg cell become DNA replication (Figure 10). Following the third cycle of dilution of the DNA methylation marks after each cycle of MET1 activity during female gametogenesis causes a gradual [12]. We propose that in addition to DME, the low level of activity is not sufficient to account for the activation of cytosine residue [16,20]. However, we showed that DME expression may require a transcriptional activator that is directly controlled by DNA methylation and DME activity.

**MEA Imprinting Is Not Primarily Controlled by MET1**

Previous studies have shown that MEA expression is regulated by MET1 and DME [16,42]. However, it remained unclear whether MET1 and DME regulate MEA imprinting directly. Genome-wide array of DNA methylation and H3K27 trimethylation have shown that the MEA locus is covered with H3K27 trimethylation but devoid of DNA methylation. DNA methylation can be found in the MEA locus only at repeats at the 3’ end of the gene, the MEA ISR domain [49–51]. However, a MEA reporter construct that does not contain the MEA ISR domain displays imprinting expression and complements the mea mutant phenotype [37] strongly suggesting that the MEA ISR is not involved in the regulation of MEA expression. Furthermore, the direct loss of DNA methylation in met1 pollen does not cause activation of the MEA paternal allele in endosperm [12], whereas MEA is activated directly by the loss of H3K27 trimethylation in mutants for Pc-G activity in both vegetative and reproductive tissues [16,17]. We do not observe any impact of the ectopic expression of MET1 by msi1 and rbr1 on MEA expression. Hence, we can conclude that MEA expression is not directly controlled by MET1. Rather, the direct silencing of MEA by H3K27 methylation implies that trimethylated H3K27 must be removed from the MEA locus during female gametogenesis to obtain transcriptional activation. It is thus possible that MET1 and DME are required directly or indirectly for the activation of a pathway that removes the H3K27 trimethylation mark from MEA leading to its activation. Alternatively, maternal MEA expression may require a transcriptional activator that is itself directly controlled by DNA methylation and DME activity.

**A Genome-Wide Demethylation during Female Gametogenesis?**

It is not clear whether DNA demethylation during female gametogenesis affects only a discrete number of loci as a result of still-unknown targeting mechanisms. Alternatively, the DNA demethylation regulated by the Retinoblastoma pathway could affect the entire genome leading to a reduction of the constitutive heterochromatin fraction in the central cell, which could be inherited in the endosperm. A global decrease of maternal DNA methylation in the endosperm was suggested to occur in maize based on the analysis of methylation-sensitive amplified polymorphism [52] and in Arabidopsis based on the reduction of the heterochromatin fraction [53]. A global demethylation is not expected to affect significantly DNA of the egg cell since it has been clearly

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**Figure 9. Msi1 Activation of Fis2 and Fwa Is Mediated through Met1**

Percentage of ovules expressing GFP or GUS activity from plants hemizygous (HE) for FIS2-GUS (A) and FWA-GFP (B) constructs in wild-type, msi1–2/+; met1–2/+; and msi1–3/+; met1–3/+ backgrounds. Error bars represent the standard deviation. The n number is represented on the top of each bar.

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established that transgenes silenced by DNA methylation remain silenced from one generation to the next [54].

Potential Conservation of Regulation of Imprinting by the Retinoblastoma Pathway

Our results provide evidence for a direct link between the Retinoblastoma pathway and genomic imprinting. The regulation of the CpG maintenance methyltransferase by the Retinoblastoma pathway is conserved between plants and mammals. Although there has been no direct evidence in mammals for a role of the Retinoblastoma protein itself in genomic imprinting, in a human cell line, the up-regulation of the expression of the DNA methyltransferase Dnmt1 caused by misregulation of pRb was correlated with an increase of CpG methylation of the paternally expressed imprinted gene Peg3 [25]. The loss of function of pRb during mouse embryonic development results in embryonic lethality. However, this lethality can be partially rescued by providing mutant embryo with a wild-type placenta, demonstrating a crucial role of pRb during placenta development. pRb deficiency in the placenta results in a decrease of nutrient transport from the mother to the embryo [55,56]. The identification of two Retinoblastoma binding proteins (Rbhp1/Arid4a and Rbhp1l1/Arid4b) that are required for the correct epigenetic marks and imprinting status at the PWS/AS domain provides further evidence for a potential role of pRb on imprinting during placenta development [57]. The data above indicate that the impact of the Retinoblastoma pathway on parental genomic imprinting in plants, that we have demonstrated may also exist in mammals and would thus provide further evidence for a convergence of imprinting between flowering plants and mammals.

Materials and Methods

Plant materials and growth conditions. Mutants fie-11, msa-6, and msi1–2 were previously characterized in our laboratory. rbr1–1/+ (SALK_012270) and rbr1–2/+ (SALK_002946) were previously described by Ebel et al. [58]. The msi1–2/+ mutant line [59] and the MSIs line were kindly provided by Lars Hennig [36]. FEs plants were previously characterized [40]. msi1–1 was described in Kaya et al. [38]. The msi1–3 line was provided by J. Paszkowski [54]. The MEA-YFP reporter line was kindly provided by R. Yadegari [37]. The KS117 line was identified after a screen in the Jim Haseloff’s enhancer trap GFP line collection [60]. The transgenic reporter lines FIS2 promoter-GUS and MEA promoter-GUS fusions constructs were kindly provided by A. Chaudhury [18]. The FWA promoter-GFP was kindly provided by T. Kinoshita [11]. The pMSI1/MSH1-mRFP1 locus fusion was generated in our laboratory [61].

Potential Conservation of Regulation of Imprinting by the Retinoblastoma Pathway

Figure 10. Model for MSI1/RBR1 Regulation of FIS2 and FWA Maternal Expression

The MSI1/RBR1 complex represses the expression of MET1 during female gametogenesis. As a result, the silencing DNA methylation marks (pink lollipops) are gradually lost during the female gamete nuclei divisions. In the central cell, DME removes the residual marks on imprinted genes such as FIS2 and FWA, resulting in their transcriptional activation. The active status is conserved on the maternal allele during endosperm development. During male gametogenesis, MET1 is expressed (Figure S7) and maintains the repression on the FIS2 and FWA paternal allele. The paternal copy remains silent during endosperm development.

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RNA extraction, cDNA preparation, and RT-PCR. Sample tissues were collected from Arabidopsis plants and frozen in liquid nitrogen. As only msi1–2/+ plants survive, we selected msi1–2 mutant seeds characterized by the overexpression of the KS117 marker at 5 d after pollination [28]. As only rbr1–1/+ plants survive, we selected rbr1–1 mutant ovules using the size difference between undeveloped rbr1–1 ovules and wild-type seeds 1 DAP before rbr1 ovules degenerate (4 DAP) [27]. RNA extraction, DNase treatment, and reverse transcription were performed as described previously [12]. Primer sequences are listed in Table S2.

Quantitative real-time RT-PCR. Real-time PCR assays were performed using Power SYBR Green PCR Master Mix (Applied Biosystems). One microliter of RT product was used to perform each PCR reaction. Amplification reaction was carried out using specific sequences are listed in Table S2.

Potential Conservation of Regulation of Imprinting by the Retinoblastoma Pathway

The specificity of the amplification was determined by performing a dissociation curve analysis. The PCR reaction and quantitative measurements were achieved with 7900HT Fast Real-Time PCR System (Applied Biosystems). Thermal cycling parameters were 2 min at 50 °C, 10 min at 95 °C, and 30 cycles of 15 sec at 95 °C, 60 sec at 60 °C. Three technical replicates were done for each sample. The ΔCt was calculated using ACT11 gene as endogenous control (Table S2). Relative quantitation (RQ) values were calculated using the 2−ΔΔCt method (RQ=2−ΔΔCt) [62]. Values given in Figure 2A represent the RQ average of five biological replicates for each point and three biological replicates (Figure 2B and 2C).

Chromatin immunoprecipitation. The ChIP experiment was performed as described previously [63] with minor modifications. Buds were ground with a pestle in liquid nitrogen and fixed with 1% formaldehyde for 10 min. The chromatin was precleared by incubating with protein A beads (Upstate) and IgY-beads (Aves Labs) respectively. After reverse cross-linking, proteinase K, and RNase treatment, the immunoprecipitated DNA was purified using a silica-gel membrane (Qiagen) and analyzed by PCR. PCR reactions were performed in 20 μl using
HotStarTag DNA Polymerase (Qiagen). Quantitative measurements of enrichment from the fragment 2 of MET1 were performed using the absolute quantitation method achieved with 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative enrichments were calculated as the percentage of the obtained values in immunoprecipitated and input fractions. Primer sequences are listed in Table S1.

GST pull-down assays. Full-length MSI1 cDNA was cloned into the BamHI site of pGEX 4T-1 vector (Amersham Pharmacia) in frame to GST. RBR1 was cloned in to the pcITE 3a (Novagen) as described [64]. Pull-down assays were performed as described previously [65]. A GST-MSI1 fusion protein was expressed in Escherichia coli BL21 cells and immobilized on glutathione agarose beads. Beads were incubated with radioactively labeled RBR1 protein followed by six consecutive washes with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], and 0.5% NP-40). Protein labeling was performed using a coupled transcription-translation system in a reticulocyte lysate system (Promega) according to the manufacturers instructions using radioabeled [35S] methionine (Amersham-Pharmacia). Following SDS/PAGE, the translated RBR1 products appeared as a major band of 120 kDa, as expected. Beads were washed and resuspended in SDS–polyacrylamide gel sample buffer (60 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 0.02% bromphenol blue, and 100 mM DTE). Samples were separated by electrophoresis on a 10% SDS–polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore). Labeled RBR1 protein was detected by autoradiography. The GST protein was detected using anti-GST antibodies (Amersham Pharmacia Cat #27457701) diluted 1:1,000 (cv).

Anti-RBR1 protein interactions in plants by bimolecular fluorescence complementation assay. Protein–protein interactions in plants were examined by BiFC assay [31]. Equal concentrations of Agrobacterium tumefaciens strain GV3101/pMP90 containing plasmids of interest (Table S3) were transiently coexpressed in Nicotiana benthamiana leaves via leaf injection procedure. Following incubation at 25 °C for 24 h, samples were examined with Leica TCS-FL confocal laser scanning microscope with 20x and 63x water immersion objectives. Image analysis was performed with Zeiss Axiosio, Zeiss CLSM-5, and Adobe Photoshop 7.0. YFP was visualized by excitation with an argon laser at 514 nm. Emission was detected with spectral detection between 525 nm and 570 nm for RBR1 and MSI1 full-length cDNA and three deletion constructs of RBR1 were cloned into the SpeI site of pS75 and pSY76 containing the N-terminal (YN), or C-terminal (YC) fragments of the YFP protein, respectively [31]. Negative controls with vectors bearing only YN or YC alone were carried out in every experiment to verify the specificity of the interactions. Each confocal section detects only a fraction of the nuclei in a given field of view, and not every cell expresses both constructs. This explains why only a fraction of cells show fluorescent nuclei in a given field of view when interaction takes place.

Microscopy and statistics. FWA-GFP, FWA-35S::ASA1, FWA-GFP, MSI1::MEAP, and MEA::YFP expression were analyzed as previously described [12,17]. Developing seeds or pistils cleared with derivative of Hoyer’s medium were observed with differential interference contrast (DIC) optics and with 20x PlanApo objective (DM6000 B, Leica). Images were acquired with a DFC290F digital camera (Nikon) and captured with using Metamorph (version 6.2, Universal Imaging Corp). In Figures 7, 8, 9, S3, S5, and S6, the error bars represent the standard deviation calculated from the mean measured per silique containing, on average, 40 ovules. The n number is represented on the top of each bar and corresponds to the total number of ovules observed.

pMSI1-MSI1-RFP and pMET1-H2B-RFP fluorescence was imaged using laser scanning confocal microscopy (Zeiss Exiter) for mRFP1 with selective settings for RFP detection (excitation, 543 nm; emission, band-pass 560 to 615 nm).

pMET1:H2B-mRFP1 plasmid construction and transformation. The pMET1 promoter was amplified by PCR using the KOD-plus-PCR kit (TOYOBO) and primer pair: R2met1-F1 and MET1-int66R (Table S2). The PCR fragment was then cloned into pENTR-D-TOPO (Invitrogen). The promoter was then fused to H2B-RFP in frame by recombination using Gateway technology (Invitrogen) into the pGreenII 6000 vector (Invitrogen) [66]. The final vector (pGreenII-pMET1-H2B-RFP) contains 947 bp upstream of the translation start site until the first 66 amino acids of MET1 in frame with H2B-RFP. Wild-type Columbia were transformed using the Agrobacterium-mediated floral dip method [67], and transgenic lines were selected on kanamycin. The presence of the transgenes was confirmed using PCR. All transgenic lines harbored consistent patterns of transgene expression. Three transgenic lines were used for the detailed observation of the expression pattern using confocal microscopy.

Supporting Information

Figure S1. Comparison of CpG DNA Methylation between Wild-Type and MSI1cs Plants

In order to know whether increased METI expression in MSI1cs leaves also leads to an increase in the amount of CpG methylation, we performed bisulfite sequencing of the methylated region of the FIS2 and FWA promoters.

(A) Analysis of CpG methylation of FIS2 promoter region using bisulfite sequencing. Twelve clones were sequenced for wild-type Col, and 11 clones were sequenced for MSI1cs.

(B) Analysis of CpG methylation of FWA promoter region using bisulfite sequencing. Eleven clones were sequenced for wild-type Col, and eight clones were sequenced for MSI1cs.

In comparison to wild-type levels, we observed in MSI1cs plants a slight increase in the methylation of the FIS2 promoter, but did not see any significant modification of the methylation level in the FWA promoter. Several factors may explain this limited effect. Increased level of METI mRNA may not result in an increased METI activity or the increase of METI activity in vegetative tissues is not sufficient to be effective. Alternatively, the targets studies may not be responsive to increased METI activity in vegetative tissues. The most likely hypothesis is that FIS2 and FWA methylated regions are already highly methylated in wild-type leaves, which prevents the observation of an increase in MSI1cs.

Methods: bisulfite-sequencing analysis was carried out with the EZ DNA Methylation-Gold Kit (Zymo Research). The treated DNA was cleaned up in accordance with the manufacturer’s instructions and used for subsequent PCR. After PCR, the products were cloned in pGem-Teasy (Promega), and individual clones were sequenced with SP6 and T7 primers. The sequences were then aligned with AlignX (Invitrogen), and the methylation level was analyzed using BiQ Analyzer (bioinformatics.bmcbioinformatics.org). Primers used for bisulfite-sequencing analyses are listed in Table S1. The ASA1 gene was used as a positive control of bisulfite chemical reaction (Kinoshita et al. [11]).

Found at doi:10.1371/journal.pbio.0060194.sg001 (928 KB EPS).

Figure S2. Specificity of the Antisense against MSI1

Antibodies against the C-terminal region of MSI1 were obtained from immunization of rabbits with the peptide MGKDEEEMGFEIERLINE (Invitrogen). A western blot was performed using serum against protein extracts from 10-d-old seedlings from Arabidopsis, wild-type Columbia, and a transgenic Columbia line carrying the gene encoding the MSI1-RFP protein fusion under the control of the MSI1 promoter (Figure 4). A major band was detected by the antibody in the wild-type background around 55 kDa, i.e., slightly above the predicted molecular weight of MSI1 (48.2 kDa). A minor band was detected at 68 kDa. In the extract of the MSI1-RFP line, an additional band was detected at 80 kDa. The molecular weight of RFP is 27 kDa, and the predicted fusion protein weight of 82 kDa corresponds to the size observed, indicating that the major protein detected by the antibody is MSI1. The intensity of the major band in the wild type was reduced, indicating a potential cosuppression effect of the expression of an additional copy of MSI1.

Found at doi:10.1371/journal.pbio.0060194.sg002 (2.37 MB EPS).

Figure S3. FIS2-GUS Expression Is Not Affected by PrC and CAF-1 Mutation

(A) Percentage of ovules or seeds expressing FIS2-GUS (homoyzogote for the marker [H0]) in wild-type (WT) and in Polycomb mutants snf-5+ and fasc-11+ before pollination (RP) and 2.5 d after pollination (2.5 DAP).

(B) Percentage of seeds expressing FIS2-GUS (hemizygote for the marker, HE) in wild-type (WT) and in CAF-1 mutant fas-1+ before pollination.

Error bars represent the standard deviation. The n number is represented on the top of each bar.

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Figure S4. RBR1/MSI1 Pathway Is Independent of DME

(A) RT-PCR on RNAs from rbr1-1–selected ovules showed no reduction of DME and MSI1 mRNA levels comparison to wild-type Col accession. GPDH is used as a control.

(B) RT-PCR on RNAs from dme-4 buds showing no reduction of RBR1 and MSI1 in dme-4 buds in comparison to wild-type C24 accession. GPDH is used as a control.

Found at doi:10.1371/journal.pbio.0060194.sg004 (440 KB EPS).
At the bicellular stage, pMET1/H2B-RFP fusion protein coexpressed with DAPI (A) pMET1/H2B-RFP is expressed in the microspore (M). (B) At the bicellular stage, pMET1/H2B-RFP expression is restricted to the generative cell (G) and is absent from the vegetative cell (V). (C) At the tricellular stage, pMET1/H2B-RFP is expressed in the two sperm cells (S). Scale bars represent 10 μm.

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