Development 140, 2953-2960 (2013) doi:10.1242/dev.092981

RNA-directed DNA methylation regulates parental genomic imprinting at several loci in *Arabidopsis*

Thiet Minh Vu[1,2], Miyuki Nakamura[3], Joseph P. Calarco[4], Daichi Susaki[5], Pei Qi Lim[1], Tetsu Kinoshita[3], Tetsuya Higashiyama[5,6], Robert A. Martienssen[4] and Frédéric Berger[1,2,*]

SUMMARY

In mammals and plants, parental genomic imprinting restricts the expression of specific loci to one parental allele. Imprinting in mammals relies on sex-dependent *de novo* deposition of DNA methylation during gametogenesis but a comparable mechanism was not shown in plants. Rather, paternal silencing by the maintenance DNA methyltransferase 1 (MET1) and maternal activation by the DNA demethylase DEMETER (DME) cause maternal expression. However, genome-wide studies suggested other DNA methylation-dependent imprinting mechanisms. Here, we show that *de novo* RNA-directed DNA methylation (RdDM) regulates imprinting at specific loci expressed in endosperm. RdDM in somatic tissues is required to silence expression of the paternal allele. By contrast, the repression of RdDM in female gametes participates with or without DME requirement in the activation of the maternal allele. The contrasted activity of DNA methylation between male and female gametes appears sufficient to prime imprinted maternal expression. After fertilization, MET1 maintains differential expression between the parental alleles. RdDM depends on small interfering RNAs (siRNAs). The involvement of RdDM in imprinting supports the idea that sources of siRNAs such as transposons and *de novo* DNA methylation were recruited in a convergent manner in plants and mammals in the evolutionary process leading to selection of imprinted loci.

KEY WORDS: *Arabidopsis*, Seed, Imprinting, Endosperm, RdDM, DNA methylation

INTRODUCTION

In mammals and plants, parental genomic imprinting restricts expression of certain loci to one parental allele (Feil and Berger, 2007). Imprinting in mammals relies on sex-dependent *de novo* deposition of DNA methylation by specific methyltransferases Dnmt3a and Dnmt3b (Ferguson-Smith, 2011; Bartolomei, 2009). The asymmetric patterns of DNA methylation on each parental allele are further maintained in the embryo by the maintenance DNA methyltransferase Dnmt1. By contrast, in flowering plants, MET1 the homolog of mammalian Dnmt1 is involved in the mechanism of parent-specific gene activation and also in maintenance of imprinted expression (Jullien and Berger, 2009). Histone methylation by Polycomb group also assists imprinting both in plants and mammals (Feil, 2009).

During plant sexual reproduction, two sperm cells fertilize two distinct female gametes, the egg cell and the central cell, producing the embryo and the endosperm, respectively (Berger and Twell, 2011). The endosperm and the embryo are surrounded and protected by the seed coat, which is of maternal sporophytic origin. Genome-wide surveys of parental allele-specific expression in *Arabidopsis*, rice and maize have identified new series of candidate imprinted genes and shown that imprinting is largely confined to genes expressed only in endosperm (Luo et al., 2011; Wolff et al., 2011; Zhang et al., 2011; Gehring et al., 2011; Hsieh et al., 2011; Waters et al., 2011). These studies show that imprinting causes maternal expression for the majority of genes but some genes are rather expressed by the paternal allele. Not only genes but also non coding RNAs might be expressed maternally (Mosher et al., 2009; Luo et al., 2011), although whether these maternal RNAs originate from seed coat or endosperm remains unknown.

MET1 maintains methylation of cytosine residues in CG contexts (Feng et al., 2010), which results in a silenced status of many imprinted genes in diploid sporophytic vegetative cells and in haploid male gametes (Jullien and Berger, 2009). The endosperm-specific expression of maternally expressed imprinted genes originates from the mechanism that activates the expression of the maternal allele. In the central cell, the transcriptional repression of MET1 (Jullien et al., 2008), together with the active demethylation by the DNA glycosylase DEMETER (Gehring et al., 2009) cause the loss of methylated CGs, resulting in transcriptional activation in the endosperm precursor: the central cell. Although a few genes imprinted in the embryo have been reported (Luo et al., 2011; Jahnke and Scholten, 2009), the origin of the mechanisms involved remains unknown.

The study of two imprinted loci in maize showed that imprinted alleles acquire DNA methylation after fertilization (Gutiérrez-Marcos et al., 2006; Jahnke and Scholten, 2009), but the origin of this methylation remained unknown. Because *de novo* methylation is unable to distinguish between two equally unmethylated parental alleles, this mechanism alone is unlikely to account for these observations. Recent studies have also suggested that mechanisms involving controls of DNA methylation other than DME and MET1 might be involved in the control of imprinting (Luo et al., 2011; Wolff et al., 2011; Zhang et al., 2011; Gehring et al., 2011; Hsieh et al., 2011; Waters et al., 2011). In addition to methylation in the CG context, plant genomes have substantial amounts of CHG and CHH methylation where H represents any base other than G (Feng et al.,

[1]Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604 Singapore. [2]Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117543 Singapore. [3]Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. [4]Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA. [5]Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan. [6]JST ERATO Higashiyama Live-Holonics Project, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan.

*Author for correspondence (fred@tll.org.sg)

Accepted 29 April 2013

2010). In plants, the DOMAINS REARRANGED METHYLTRANSFERASES (DRM) methylate cytosine residues in the CHH context (Cao and Jacobsen, 2002). CHH methylation by DRM is associated with the RNA-directed DNA methylation pathway (RdDM). RdDM is initiated by the production of the 24-nucleotide small interfering RNAs (siRNAs) by a pathway involving the plant-specific RNA polymerases PolIV and PolV (Cao et al., 2003; Pontier et al., 2005; Daxinger et al., 2009). The major subunits of RNA POLIV and POLV, are NRPD1a and NRPD1b, respectively, and NRPD2A is the second largest subunit of RNA POLIV and POLV. Thus far, there has been no direct evidence that the RdDM pathway controls imprinting by DNA *de novo* methylation in plants. Here, we dissect the expression of various members of the RdDM pathway in *Arabidopsis* gametes and demonstrate that the RdDM pathway participates in parental genomic imprinting.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana ecotypes Columbia-0 (Col-0), Wassilewskija (Ws), Landsberg *erecta* (L*er*-0), Cape Verde Islands (Cvi-0), RLD and C24 were obtained from the *Arabidopsis* Stock Centre (http://www.arabidopsis.org). The *nrpd2a-1* (SALK_095689) was received from C. Pikaard's Lab (Onodera et al., 2005). The mutant seeds *nrpd1b-12* (SALK_033852), *nrpd1a-4* (SALK_083051), *drm1-2* (SALK_031705), *drm2-2* (SALK_150863), double homozygous *drm1, drm2, cmt3-11t* (SALK_148381C), *sdc* (SALK_017593) and *mop9.5* (FLAG_462F03, FLAG_508H08) were obtained from ABRC. Primers for genotyping are described in supplementary material Table S1. MET1 antisense (MET1a/s) was characterized by J. Finnegan (Finnegan et al., 1996). Reduction of 70% CpG methylation was reported in this line and the effect is dominant in vegetative tissues. The *MET1*/*met1-3* (*met1-3*/+) line was provided by J. Paszkowski (Saze et al., 2003). The FIE co-suppressor (*FieCoS*) line has previously been characterized by N. Ohad's group (Katz et al., 2004). The mutant *dme11* has been characterized previously (Guitton et al., 2004).

Analyzing allele-specific expression of imprinted genes

The origin of parental transcripts of imprinted genes was analyzed by RT-PCR followed by enzymatic digestion or sequencing. Seeds or siliques from the reciprocal crosses between two selected ecotypes harboring the nucleotide polymorphism were used for RNA extraction (RNeasy Plant Minikit, QIAGEN) and reverse transcription. Transcripts were amplified by Illustra Taq polymerase (GE) in 20 μl PCR reaction for 35 cycles with specific primers that span the polymorphism sites. Different parental alleles were distinguished by using restriction enzymes (NEB) that recognize the restriction sites introduced by the polymorphism. For allele-specific RT-PCR analysis by sequencing, PCR was performed using HOTSTART Taq polymerase (QIAGEN) in 40 μl reaction. Prior to sequencing (BigDye Terminator Kit, ABI), PCR products were purified by MinElute column (QIAGEN) or gel purification (if required) using the Gel DNA Recovery Kit (Zymo Research). Sequencing results were analyzed by SeqMan (Lasergene).

RT-PCR and quantitative PCR

Unfertilized ovules obtained from ten emasculated pistils were used for RNA isolation using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA). The RNeasy Micro Kit (QIAGEN) was used to isolate total RNA from seeds. RNA isolation from siliques and leaves was carried out using the RNeasy Mini Kit (QIAGEN). The same amount of total RNAs from different tissues was used in reverse transcriptase reaction reactions using Stratagene transcriptase (Agilent Technologies). Quantitative PCR was performed with the ABI Prism 7900 HT Fast System using SYBR Green PCR Master Mix (Applied Biosystems) according to supplier's recommendations. Relative quantitative values were calculated by comparative C_T method (Schmittgen and Livak, 2008). The s.d. was calculated based on at least three technical replicates and two biological replicates.

Isolation of female gametes and RT analysis

Isolation of female gametophytic cells was performed as described previously (Ikeda et al., 2011). In detail, the total RNA was isolated from 10 egg cells, five central cells, 10 synergid cells and one ovary using following kits: Dynabeads mRNA DIREC Micro Kit for female gametes and Ambion RNAqueous-Micro for the ovary. The RT-PCR results were reproduced on two biological replicates using Ex Taq (TaKaRa) in 20 μl reaction with 45 cycle amplification.

Plasmid construction and transgenic plants

Cloning was performed using GATEWAY technology (Invitrogen). The *pSDC-H2B-RFP* contains 2731 bp promoter sequences of *SDC* (At2g17690) locus in order to drive the expression of HISTONE2B-RFP in the plasmid pGnk-GW-H2B-RFP (Ingouff et al., 2009). Fifteen T1 transgenic lines displayed the consistent fluorescent expression pattern. The reporter transgenic line p*FWA-GFP* has been described previously (Kinoshita et al., 2004).

Bisulfite genomic DNA sequencing

Endosperm and embryos dissected for bisulfite sequencing were described previously (Ikeda et al., 2011). In detail, embryos and endosperm were isolated from seeds produced by reciprocal crosses between Cvi and RLD carrying SNPs, which were used to differentiate the parental alleles (supplementary material Fig. S4). Sperm cells were sorted through via FACS as published previously (Borges et al., 2008) and the extracted DNA was fragmented, treated with sodium bisulfite and sequenced using next generation sequencing (Lister et al., 2008; Calarco et al., 2012). Levels of methylation were calculated at each cytosine as the ratio C/C+T, and only residues with at least four reads mapping to them were considered. Methylation profiles were displayed on separate tracks within each cytosine context (CG, CHG and CHH) using the Integrated Genome Viewer (IGV).

RESULTS

Identification of imprinted genes controlled by the RdDM pathway

From the databases of candidate imprinted genes in *Arabidopsis* (Hsieh et al., 2011; Gehring et al., 2011; Wolff et al., 2011), we selected five maternally expressed genes that could be silenced by the RdDM pathway in vegetative tissues and expressed in endosperm according to databases available online (Le et al., 2010). Using single nucleotide polymorphisms we confirmed that *SUPPRESSOR OF drm1 drm2 cmt3* (*SDC*), *MOP9.5* (At5g24240), At3g21830, At1g61090 and At2g34880 are maternally expressed (Fig. 1A-C) and that their expression is repressed by RdDM and MET1 in vegetative tissues (Fig. 1D-G), suggesting that these five genes are maternally expressed imprinted genes controlled by RdDM. However, inheritance of maternal transcripts from the female gametes also lead to maternal expression, although no active transcription of the maternal allele takes place after fertilization. We thus investigated further the spatial and temporal paternal of expression of the five candidate imprinted genes.

In order to visualize *SDC* expression, we obtained a transcriptional reporter line that expresses the fluorescent protein fusion HISTONE2B-RFP (H2B-RFP) under the control of the *SDC* promoter. According to the pattern of expression of H2B-RFP, *SDC* was expressed in endosperm but not in seed coat in wild-type background (supplementary material Fig. S1B,E). By contrast, *SDC* was expressed in the seed coat in *nrpd2a-1* homozygous mutant (supplementary material Fig. S1F), confirming the transcriptional repression of *SDC* by RdDM in diploid vegetative tissues. In wild-type plants carrying *pSDC::H2B-RFP*, we observed that *SDC* is expressed in the central cell but not in the egg cell (Fig. 2A,B). We

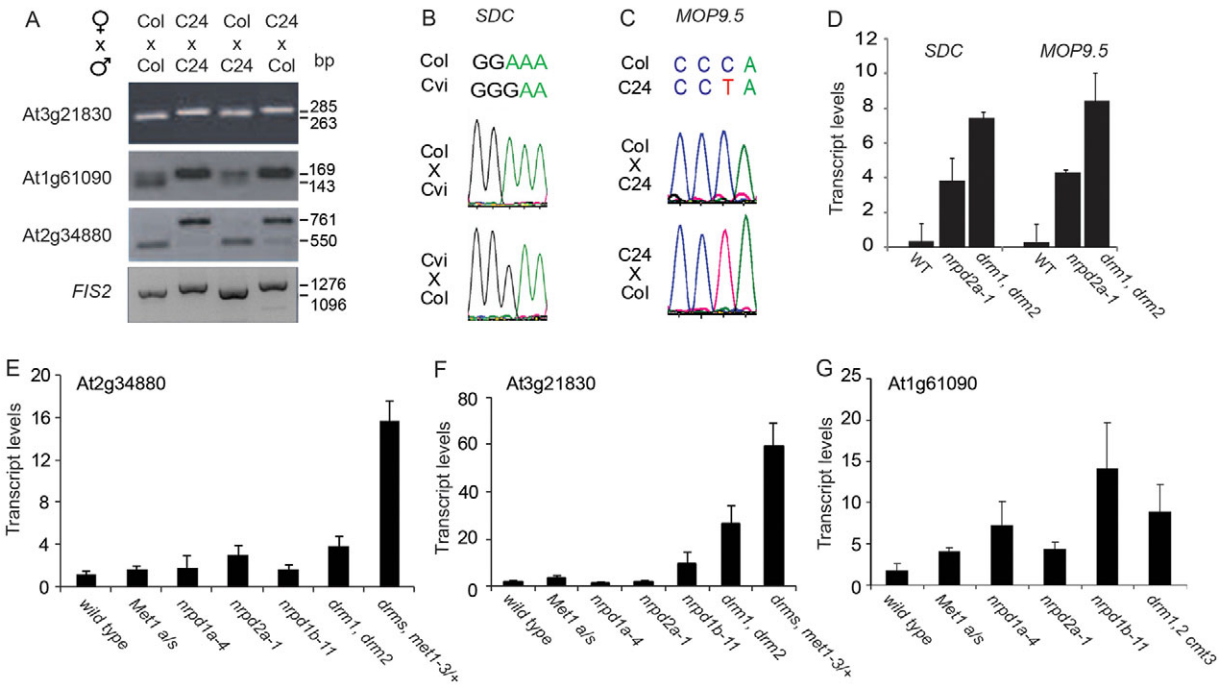

Fig. 1. Potential imprinted genes regulated by the RdDM pathway. (**A**) Allele-specific RT-PCR analysis showed the maternal expression of At1g61090, At2g34880 and At3g21830. (**B**,**C**) Maternal expression of (B) *SDC* and (C) *MOP9.5* was confirmed by RT-PCR sequencing chromatographs at selected SNP present in different accessions. RNA was extracted 3 days after pollination (DAP) from seeds obtained from reciprocal crosses between two different accessions containing single nucleotide polymorphisms (SNPs), which allow maternal and paternal expression of the genes to be distinguished. SNPs were recognized by enzyme restriction digestion or sequencing. Primer sequences and restriction enzymes used are provided in supplementary material Table S1. The maternally expressed gene *FIS2* was used as a positive control. (**D-G**) qRT-PCR analysis of expression of candidate imprinted genes in seedlings from mutants in RdDM and MET1 pathways. *drms* strands from *drm1*, *drm2*. The qPCR results were normalized by *ACT11* using three technical replicates and two biological replicates. Error bars indicate s.d. The *y*-axis represents levels relative to the *ACT11* control.

did not observe *SDC* expression in sperm cells (Fig. 2E,F). After fertilization, we detected expression of *SDC* and *pSDC::H2B-RFP* in endosperm but not in the embryo (Fig. 2C,D,G). We observed an increase of *SDC* transcripts levels by twofold in 2 DAP (days after pollination) seeds compared with flower buds, and a 5-fold increase in 4 DAP seeds compared with flower buds (supplementary material Fig. S1B,C). These results indicated that *SDC* transcripts in endosperm did not originate from the central cell and that *SDC* was expressed in endosperm. The maternal expression of *SDC* was observed in crosses between different natural accessions (Fig. 2H; supplementary material Fig. S1A). These results confirmed that *SDC* is a maternally expressed imprinted gene expressed in endosperm.

MOP9.5 was also expressed in endosperm (Fig. 3A). Levels of *MOP9.5* expression were too low to allow detection using a transcriptional reporter similar to that used for *SDC* (not shown). Allele-specific RT-PCR showed that only the maternal allele of *MOP9.5* was expressed in seeds (Fig. 3B). Real-time PCR showed that levels of *MOP9.5* maternal transcripts increased after fertilization (Fig. 3C), indicating that *MOP9.5* was expressed after fertilization and that *MOP9.5* is a maternally expressed imprinted gene.

Similar increasing levels of expression after fertilization were observed for At1g16090, At2g34880 and At3g21830 (supplementary material Fig. S2), indicating that these genes are expressed actively after fertilization and that their maternal expression does not reflect inheritance of maternal transcripts from the female gamete. However, the expression levels of the maternally expressed imprinted genes At3g21830, At1g61090 and At2g34880 were much lower than *SDC* and *MOP9.5*. We thus focused further work on *SDC* and *MOP9.5* as models to study whether RdDM plays a role in imprinting.

RdDM silences the paternal allele of imprinted genes

SDC was not expressed in pollen (Fig. 2E,F) and, accordingly, we reported that in sperm cells DNA methylation is found in all contexts, including CHH in the region containing the *SDC* promoter (Calarco et al., 2012). This observation suggested that *SDC* expression is repressed by *de novo* DNA methylation in male gametes, but the effect of RdDM on the expression of the paternal allele of *SDC* in endosperm remained unknown. To address this issue we used Col and Cvi accessions to distinguish the parental alleles of *SDC* and crossed wild-type ovules with pollen from mutants for RdDM. In seeds from such crosses, we observed ectopic transcriptional activity of the endogenous *SDC* paternal allele (Fig. 4A). We also observed expression of the paternal allele of the *pSDC::H2B-RFP* reporter when paternally inherited from *nrpd2a* (Fig. 4B). These data show that the RdDM pathway is involved in silencing the *SDC* paternal allele that is inherited in endosperm.

In leaves and in sperm cells, a domain in the 5′ region of *MOP9.5* that overlaps with the 3′ of the coding sequence of the neighbor gene *At5g24250* was methylated in all contexts (supplementary material Fig. S3A), also suggesting that *de novo* methylation participated in the silencing of *MOP9.5* in male gametes. In seeds produced by crosses between wild-type ovules and pollen of mutants affected for

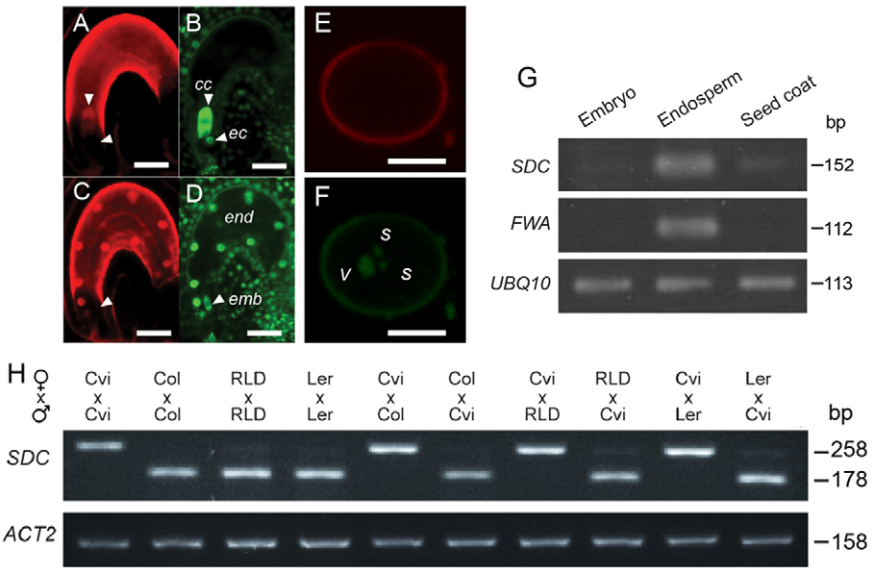

Fig. 2. Expression pattern of *SDC*. (**A-F**) Expression of HISTONE 2B fused to the MONOMERIC RED FLUORESCENT PROTEIN under the control of the *SDC* promoter (*pSDC::H2B-RFP)* in (A) ovules before fertilization, (C) seeds at 2 DAP and (E) mature pollen. (B,D,F) Confocal sections corresponding to the sections shown in A, C and E, respectively, show GFP signal reporting the expression of DNA LIGASE1, which marks all nuclei in ovules and seeds. The red signal in the cell wall surrounding the embryo sac (A) and endosperm (C) originates from autofluorescence. cc, central cell nucleus; ec, egg cell nucleus; s, s sperm nuclei; v, vegetative nucleus; end, endosperm; emb and arrowheads, embryo nuclei (confocal sections; RFP channel for A and C; GFP channel for B and D). Scale bars: 30 μm. (**G**) RT-PCR analysis of *SDC* expression in different parts of the developing seed (7 DAP), *FWA* is the positive control for the endosperm-specific expression; *UBQ10* is the loading control. (**H**) Allele-specific RT-PCR analysis of *SDC* in reciprocal crosses among different accessions. RT-PCR was performed on total RNA extracted from 4 DAP seeds produced by reciprocal crosses between Cvi and the other accessions, including Col, RLD and L*er*. *ACT2* was used as a loading control.

the RdDM pathway, we observed ectopic activation of the paternal *MOP9.5* allele (Fig. 4C). We concluded that the imprinted expression of *MOP9.5* also depends on the RdDM pathway that silences the paternal allele of this gene in endosperm.

However, the levels of CHH methylation at *SDC* and *MOP9.5* promoters were lower in male gametes than in leaves (Calarco et al., 2012) (supplementary material Fig. S3A), questioning whether RdDM activity in male gametes silenced *SDC* and *MOP9.5*, effectively. In order to address this question, we crossed wild-type ovules with pollen from *nrpd2a*/+ plants that experience RdDM activity during vegetative development and lose RdDM activity only during gametogenesis. In endosperm from these crosses, we observed that both *SDC* and *MOP9.5* remained fully imprinted with no detectable expression from the paternal allele (supplementary material Fig. S3B,C). In summary, we did not observed activation of *SDC* and *MOP9.5* paternal alleles from *nrpd2a* pollen from *nrpd2a*/+ heterozygous plants, but we did observe activation of *SDC* and *MOP9.5* paternal alleles from *nrpd2a* homozygous plants that inherit a genome from somatic sporophytic tissues deprived of NRPD2a. We thus concluded that RdDM was required in sporophytic diploid paternal tissues but not in the gametophytic male haploid germline to silence *SDC* and *MOP9.5* paternal alleles. Hence, our results suggest that effective silencing of imprinted genes by RdDM takes place primarily prior to gametogenesis.

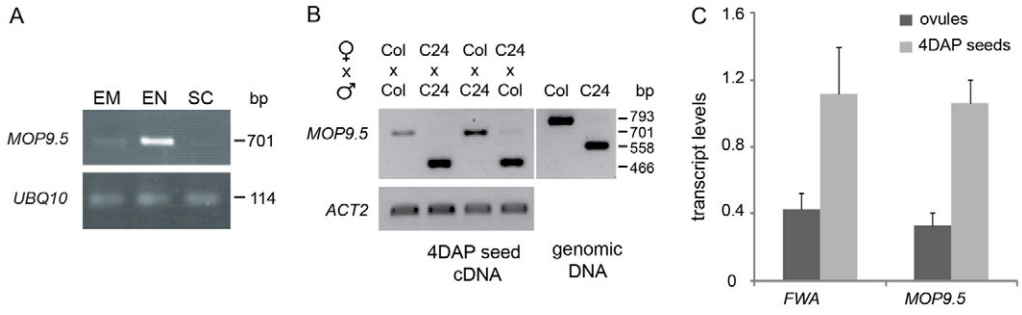

Fig. 3. Maternal expression of *MOP9.5* in endosperm. (**A**) RT-PCR analysis of *MOP9.5* expression in endosperm, embryo and seed coat isolated from 7 DAP seeds. (**B**) Allele-specific RT-PCR analysis of *MOP9.5* expression in seeds produced by reciprocal crosses between Col and C24. RT-PCR was performed on total RNA extracted from 4 DAP seeds. Primers used to amplify *MOP9.5* span an intron, therefore PCR products from genomic DNA and cDNA are different sizes. *ACT2* is the loading control. (**C**) qRT-PCR analysis of expression levels of *MOP9.5* and *FWA* in unfertilized ovules and 4 DAP seeds. qPCR results showed that levels of transcripts of MOP9.5 increased nearly three times in 4 DAP seeds compared with unfertilized ovules. Error bars indicate s.d.

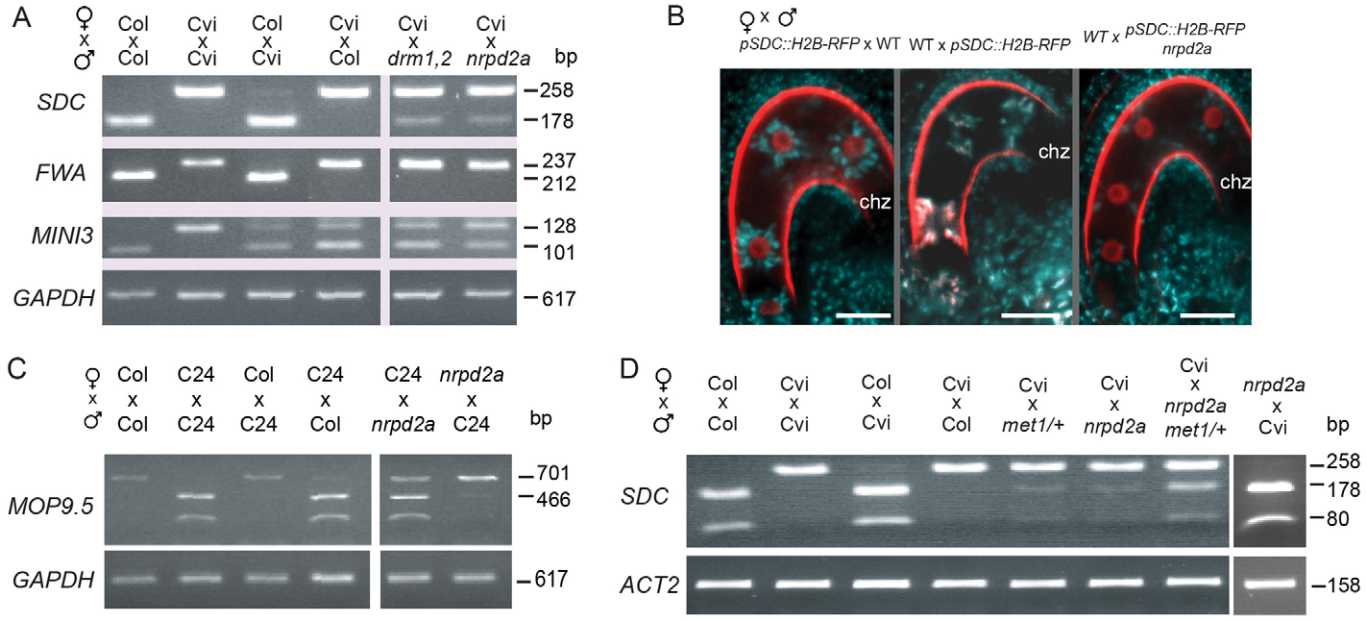

Fig. 4. RdDM silences the paternal alleles of imprinted genes. (**A**) Allele-specific RT-PCR analysis of parental origin of *SDC* transcripts. *FWA* and *MINI3* are positive controls for maternal expression and bi-parental expression, respectively; *GAPDH* is the loading control. Total RNA was extracted from 2 DAP seeds. (**B**) The RFP patterns report the expression of *SDC* in in seeds from crosses between ovules carrying *pSDC::H2B-RFP* and wild-type pollen; and a wild-type mother plant crossed with pollen carrying *pSDC::H2B-RFP*; a wild-type mother plant crossed with *nrpd2a* pollen carrying *pSDC::H2B-RFP*. (confocal sections, light blue reports background auto-fluorescence from plastids that outlines endosperm nuclei; the red signal in the cell wall surrounding the endosperm originates from auto-fluorescence). In the central panel, red auto-fluorescence from bleached chloroplasts around endosperm was observed. chz marks the chalazal pole of endosperm. Scale bars: 20 µm. (**C**) Allele-specific RT-PCR analysis of *MOP9.5* expression in seeds produced by reciprocal crosses between C24 × Col and C24 × *nrpd2a-1*, *GAPDH* was used as the loading control. (**D**) Allele-specific RT-PCR analysis of *SDC* expression in seeds from reciprocal crosses between Col and Cvi, and a Cvi mother crossed with a single mutant in the Col background, with *met1-3*/+, with *nrpd2a* and with the double mutant *nrpd2a/met1-3*/+. *ACT2* is the loading control.

Moreover, CG methylation by *MET1* participates in the extension of DNA methylation from the tandem repeats towards the transcription start site (TSS) and in silencing of *SDC* paternal allele (Henderson and Jacobsen, 2008). Accordingly, the paternal *SDC* allele contributed by *met1*/+ plants was ectopically expressed at low levels (Fig. 4D; supplementary material Fig. S3D). We further showed a stronger ectopic expression of the *SDC* paternal allele from crosses between wild-type ovules and pollen from *nrpd2a/nrpd2a*; *met1*/+ plants (Fig. 4D; supplementary material Fig. S3D). These results indicate that silencing of the paternal allele of *SDC* silenced by RdDM requires the joint activities of maintenance methylation by MET1 during male gametogenesis and *de novo* methylation by RdDM during sporophytic development.

Maternal expression of *SDC* and *MOP9.5*

In order to gain insight into the mechanism responsible for maternal activation of *SDC* and *MOP9.5*, we tested whether DEMETER, the DNA glycosylase that participates in maternal activation of several imprinted genes was also involved in *SDC* imprinting. We detected a marked decrease of *SDC* expression in seeds deficient for DME (Fig. 5A). By contrast, *MOP9.5* expression in seeds did not depend on DME (Fig. 5A). These data suggested that another mechanism in female gametes was sufficient to activate the expression of *MOP9.5*. Among the DNA methyltransferases, only low levels of DRM2 were detected in the central cell (Jullien et al., 2012). DRM2 activity relies on RNA polymerases PolIV and PolV, which are essential for the production and use of siRNAs, respectively (Herr et al., 2005; Onodera et al., 2005; Mosher et al., 2008; Daxinger et al., 2009). We isolated central cells and the associated synergid cells from *Arabidopsis* ovules (Ikeda et al., 2011; Gebert et al., 2008), and analyzed the presence of transcripts for genes controlling POLIV and POLV activity. We could not detect *NRPD1a*, *NRPD1b* or *NRPD2a* transcripts by RT-PCR in these cells (Fig. 5B). Our results suggest that in addition to the low levels of *DRM2*, the absence of PolIV and POLV major subunits prevents active RdDM in the central cell. The joint repression of the DNA methyltransferases activity is sufficient to activate expression of RdDM targets in sporophytic tissues (Fig. 1) and thus is also likely

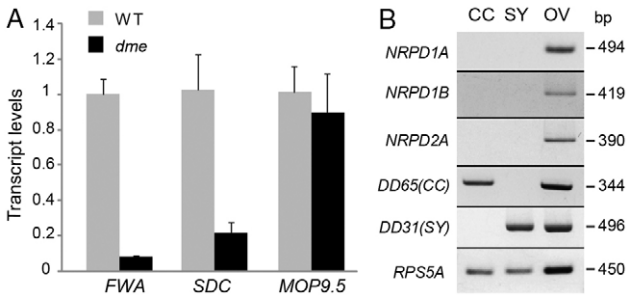

Fig. 5. Origin of the activation of maternal alleles. (**A**) RT-qPCR analysis of *SDC* and *MOP9.5* expression in *dme* mutant and wild-type seeds. *FWA* is used as the positive control. The transcripts levels were normalized against *ACTIN11* (*ACT11*) transcripts levels in wild-type seeds. Error bars indicate s.d. (**B**) Expression of the PolIV/PolV major subunits in isolated female gametophytes. *DD65* and *DD31* were used as markers for central cells (CC) and synergid cells (SY) isolated from ovules (OV). *RPS5A* is used as the loading control.

to be sufficient to activate the maternal allele of *MOP9.5* and *SDC* in the central cell. The moderate reduction of the levels of *SDC* transcripts by *dme* loss of function (Fig. 5A) also suggests that low RdDM and MET1 activities in the central cell assist DME to express *SDC* in the central cell, leading to inheritance of an active maternal allele in endosperm.

Maintenance of imprinted expression of *SDC* and *MOP9.5* in the endosperm

To determine whether the pattern of DNA methylation at the imprinted *SDC* locus paralleled the expression of the two parental alleles of *SDC* after fertilization, we performed bisulfite sequencing of DNA extracted from isolated endosperm where *SDC* is expressed and from isolated embryos that do not express *SDC*. The promoter of *SDC* contains seven 32 bp tandem repeats, which produce siRNAs and are methylated by the RdDM pathway in vegetative tissues (supplementary material Fig. S1A) (Henderson and Jacobsen, 2008; Numa et al., 2010). We analyzed DNA methylation at the transcription start site (TSS) and in the 5′ upstream region containing 32 bp repeats (Fig. 6A; supplementary material Fig. S1A, Figs S4, S5). In embryos, both *SDC* parental alleles were methylated with a comparable profile. By contrast in endosperm, we observed a marked reduction of CG but not CHH methylation on the maternal allele of *SDC* in repeats and at the TSS, compared with the paternal allele (Fig. 6B; supplementary material Fig. S4B), which correlated with the maternal-specific expression of *SDC* in the endosperm. Overall, our data suggest that, in endosperm, MET1 maintains the difference of methylation in CG contexts between the parental alleles, leading to the maintenance of maternal-specific expression of imprinted genes *SDC* and *MOP9.5*.

Recent studies reported the expression of genes involved in RdDM in developing seeds. During the early syncytial phase of endosperm development, which encompasses the period when *SDC* and *MOP9.5* showed high levels of imprinted expression, endosperm showed low levels of expression of *NRPD2a* and other major components of the RdDM pathway: *NRPD1b*, the RNA DEPENDENT RNA POLYMERASE 2 (RDR2), DICERLIKE 3 (DCL3), ARGONAUTE 4 (AGO4) (Belmonte et al., 2013) and of DMR2 (Jullien et al., 2012). The low levels of expression of main components of the RdDM pathway in endosperm are compatible with the fact that both parental alleles of *SDC* do not become silenced, which would be the case if RdDM activity were high. The expression of RdDM related genes increases markedly after endosperm cellularization (Belmonte et al., 2013), which probably explains why we observed similar CHH methylation profiles on both parental alleles of *SDC* in cellular endosperm (Fig. 6A,B).

SDC and *MOP9.5* are not essential for endosperm development

We hypothesized that the dedicated complex transcriptional mechanisms that lead to maternal expression of *SDC* and *MOP9.5* in endosperm was selected because these genes play important functions, as shown for other imprinted genes (Berger et al., 2013). In order to study SDC and MOP9.5 function in endosperm, we isolated two null *sdc* and *mop9.5* alleles (Fig. 6C,D). In absence of *SDC* or *MOP9.5* expression, endosperm development was not affected (Fig. 6E), and null *sdc* and *mop9.5* plants produced viable seeds similar to wild type (not shown). Hence, SDC and MOP9.5 functions are not essential for endosperm development.

DISCUSSION

Our study shows that several loci that are repressed by RdDM in somatic vegetative tissues are maternally expressed in endosperm. The repression of these loci also involves MET1. MET1 maintains DNA methylation in somatic tissues and male gametes, whereas we show that RdDM does not participate to the maintenance of the DNA methylation in the male gametes. In the central cell, the low expression of several key genes involved in RdDM together with the low activity of MET1 and other DNA methyltransferases (Jullien et al., 2008; Jullien et al., 2012) is expected to contribute to the

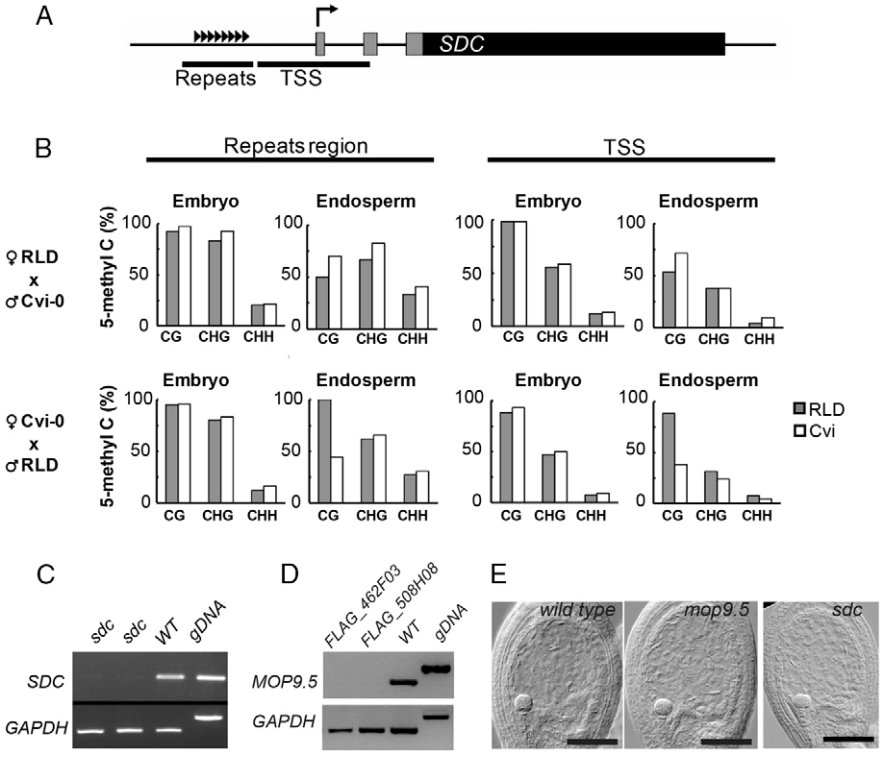

Fig. 6. Maternal maintenance of imprinted genes in endosperm. (**A**) The 5′ upstream region and transcriptional start site (TSS) of *SDC*. Triangles represent the 32 bp tandem repeat sequences. Gray boxes and the black box represent the 5′-UTR exon and coding sequence of *SDC*, respectively. (**B**) Percentage methylation of the 32 bp repeat region (left) and of the −247 bp to +206 bp region relative to the predicted TSS (right) in the embryo and endosperm (7 DAP). DNA isolated from dissected embryos and endosperm that had been crossed between RLD and Cvi were subjected to bisulfite sequencing (n=12). Conserved cytosine residues both in RLD and Cvi shown in supplementary material Fig. S4 were used for the analysis. (**C**,**D**) RT-PCR analysis of *SDC* (C) and *MOP9.5* (D) expression in 3 DAP seeds collected from wild-type and mutant plants for *SDC* and *MOP9.5*. *GAPDH* is the loading control. (**E**) Light microscope pictures of wild-type seeds, *mop9.5* (FLAG_508H08) and *sdc* (SALK_017593) in the seed at the globular embryo stage. Seeds were cleared by chloral hydrate (DIC microscopy). Scale bars: 100 µm.

activation of the maternal allele of the imprinted genes studied. In the case of some imprinted genes but not all of them, DME is also required for maternal activation. After fertilization, RdDM activity is limited, if not absent, during the early phase of endosperm development and, during this period, MET1 appears to be the main pathway responsible for maintenance of the different levels of DNA methylation between the parental alleles. We thus conclude that *de novo* methylation assists MET1 in the maternal expression of imprinted genes in plants.

De novo methylation relies on the production of 24-nucleotide non-coding siRNAs. The likely involvement of non-coding piRNAs in *de novo* DNA methylation in mammals (Law and Jacobsen, 2010) and the demonstration that small RNAs regulate one imprinted locus in mice (Watanabe et al., 2011) further support the idea of convergent evolution of imprinting mechanisms in plants and mammals (Feil and Berger, 2007). Yet in *Arabidopsis*, removal of DNA methylation creates the parent-specific expression, whereas in mammals imprinting is created by deposition of DNA methylation in a parent-specific manner (Ferguson-Smith, 2011; Bartolomei, 2009). In plants, the opposition of DNA methylation patterns between male and female gametes appears to be sufficient to create a blueprint that causes imprinted expression at specific loci. Hence, the maternal expression in the endosperm becomes a mere consequence of the differential expression in the central cell and sperm cells. By contrast, in mammals, imprinted expression is not reflected by differential expression in gametes and becomes apparent only after fertilization.

Non-coding RNAs of maternal origin have been detected from fractions containing endosperm and seed coat, and it has been proposed that these non-coding RNAs might be expressed only from the maternal allele (Mosher et al., 2009). Collectively, our study and others (Jullien et al., 2012; Belmonte et al., 2013) indicate that early endosperm development (before 7 DAP in *Arabidopsis*) is marked by low activity of RdDM. This is in agreement with the fact that the maternal allele of imprinted genes is not targeted and silenced by RdDM activity during early endosperm development. Expression of RdDM components appears to peak after 7-10 DAP in endosperm (Belmonte et al., 2013), which corresponds to the developmental stage of seeds used by Mosher et al. (Mosher et al., 2009) and could explain the maternal origin of the non-coding RNAs detected from materials containing endosperm and seed coat used in this study. Yet the mechanism of production of endosperm non-coding RNAs and their roles remain unclear (Mosher et al., 2011).

The association between transposable elements (TEs) and several imprinted loci in plants and in mammals suggests that TEs play a role in establishing imprinting expression (Kaneko-Ishino and Ishino, 2010; McCole et al., 2011; Wolff et al., 2011; Walter and Paulsen, 2003; Wilkins and Haig, 2003; Okamura et al., 2004; Fujimoto et al., 2008; Hsieh et al., 2011). This hypothesis is further supported by the fact that DME might have a preference for de-methylating TEs (Gehring et al., 2009). Although it is now apparent that DME is also expressed in cell types other than the central cell (Schoft et al., 2011) and is dispensable for imprinting of certain loci (this study) (Jullien et al., 2008; Tiwari et al., 2008; Hsieh et al., 2011; Gehring et al., 2011). TEs are rather silenced by RdDM (Saze and Kakutani, 2007; Martienssen et al., 2008; Mirouze et al., 2009), which makes MET1 unlikely to be involved in the initial silencing event required in creating a new imprinted locus. Our findings suggests that any event (not only TE insertion) that leads to production of siRNA and is sufficient to target *de novo* DNA methylation and silencing, may also be sufficient to prime imprinted expression in endosperm. This would explain the origin of many imprinted genes, which are not associated with a TE (Gehring et al., 2011; Hsieh et al., 2011). The absence of impact of the loss of *SDC* and *MOP9.5* on endosperm suggests that the primary selection of imprinting genes rather relies on advantages conferred by their silencing in sporophytic vegetative tissues. A recent survey shows that ectopic expression of imprinted genes during vegetative development is deleterious (Berger et al., 2013). We propose that the evolution of imprinted genes is initiated by silencing of a locus by RdDM, which is beneficial for vegetative development. As a result of the absence of RdDM activity, such a locus is activated in the central cell, while the silenced state in maintained in sperm cells. The differential epigenetic status of the locus is inherited in endosperm after fertilization, as long as the site methylated by RdDM can be maintained by MET1. We thus propose that the peculiar control of RdDM activity in plant gametes provides a mechanism that primes evolution of imprinted loci.

Acknowledgements
We dedicate this manuscript in memory of Simon Chan who provided critical reading of this manuscript, and we thank Professor David Baulcombe for providing seeds of *nrpd2a*, *nrpd2b* mutants.

Funding
F.B., P.Q.L. and T.M.V. were supported by Temasek Lifesciences Laboratory and Department of Biological Sciences at National University of Singapore. M.N. was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry [BRAIN 03-01 to T.K.]. T.H. was supported by JST ERATO Higashiyama Live-Holonics Project and D.S. was supported by a grant [number 7811] from the Japan Society for the Promotion of Science Fellowships.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
M.N. performed the analyses reported in Fig. 2G, Fig. 3A and Fig. 6B. J.P.C. provided Fig. S3A. D.S. provided Fig. 5B. T.M.V. performed all other experiments with technical assistance from P.Q.L. F.B. provided help with confocal microscopy. T.M.V. and F.B. wrote the manuscript with crucial advice from T.K., T.H. and R.A.M.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.092981/-/DC1

References

Bartolomei, M. S. (2009). Genomic imprinting: employing and avoiding epigenetic processes. *Genes Dev.* **23**, 2124-2133.
Belmonte, M. F., Kirkbride, R. C., Stone, S. L., Pelletier, J. M., Bui, A. Q., Yeung, E. C., Hashimoto, M., Fei, J., Harada, C. M., Munoz, M. D. et al. (2013). Comprehensive developmental profiles of gene activity in regions and subregions of the Arabidopsis seed. *Proc. Natl. Acad. Sci. USA* **110**, E435-E444.
Berger, F., Vu, M.T., Li, J. and Chen, B. (2013). Hypothesis: Selection of imprinted genes is driven by silencing deleterious gene activity in somatic tissues. *Cold Spring Harb. Symp. Quant. Biol.* **77**, doi 10.1101/sqb.2012.77.014514.
Berger, F. and Twell, D. (2011). Germline specification and function in plants. *Annu. Rev. Plant Biol.* **62**, 461-484.
Borges, F., Gomes, G., Gardner, R., Moreno, N., McCormick, S., Feijó, J. A. and Becker, J. D. (2008). Comparative transcriptomics of Arabidopsis sperm cells. *Plant Physiol.* **148**, 1168-1181.
Calarco, J. P., Borges, F., Donoghue, M. T., Van Ex, F., Jullien, P. E., Lopes, T., Gardner, R., Berger, F., Feijó, J. A., Becker, J. D. et al. (2012). Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA. *Cell* **151**, 194-205.
Cao, X. and Jacobsen, S. E. (2002). Role of the arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing. *Curr. Biol.* **12**, 1138-1144.
Cao, X., Aufsatz, W., Zilberman, D., Mette, M. F., Huang, M. S., Matzke, M. and Jacobsen, S. E. (2003). Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Curr. Biol.* **13**, 2212-2217.

Daxinger, L., Kanno, T., Bucher, E., van der Winden, J., Naumann, U., Matzke, A. J. and Matzke, M. (2009). A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. *EMBO J.* **28**, 48-57.
Feil, R. (2009). Epigenetic asymmetry in the zygote and mammalian development. *Int. J. Dev. Biol.* **53**, 191-201.
Feil, R. and Berger, F. (2007). Convergent evolution of genomic imprinting in plants and mammals. *Trends Genet.* **23**, 192-199.
Feng, S., Jacobsen, S. E. and Reik, W. (2010). Epigenetic reprogramming in plant and animal development. *Science* **330**, 622-627.
Ferguson-Smith, A. C. (2011). Genomic imprinting: the emergence of an epigenetic paradigm. *Nat. Rev. Genet.* **12**, 565-575.
Finnegan, E. J., Peacock, W. J. and Dennis, E. S. (1996). Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development. *Proc. Natl. Acad. Sci. USA* **93**, 8449-8454.
Fujimoto, R., Kinoshita, Y., Kawabe, A., Kinoshita, T., Takashima, K., Nordborg, M., Nasrallah, M. E., Shimizu, K. K., Kudoh, H. and Kakutani, T. (2008). Evolution and control of imprinted FWA genes in the genus Arabidopsis. *PLoS Genet.* **4**, e1000048.
Gebert, M., Dresselhaus, T. and Sprunck, S. (2008). F-actin organization and pollen tube tip growth in Arabidopsis are dependent on the gametophyte-specific Armadillo repeat protein ARO1. *Plant Cell* **20**, 2798-2814.
Gehring, M., Reik, W. and Henikoff, S. (2009). DNA demethylation by DNA repair. *Trends Genet.* **25**, 82-90.
Gehring, M., Missirian, V. and Henikoff, S. (2011). Genomic analysis of parent-of-origin allelic expression in Arabidopsis thaliana seeds. *PLoS ONE* **6**, e23687.
Guitton, A. E., Page, D. R., Chambrier, P., Lionnet, C., Faure, J. E., Grossniklaus, U. and Berger, F. (2004). Identification of new members of Fertilisation Independent Seed Polycomb Group pathway involved in the control of seed development in Arabidopsis thaliana. *Development* **131**, 2971-2981.
Gutiérrez-Marcos, J. F., Costa, L. M., Dal Prà, M., Scholten, S., Kranz, E., Perez, P. and Dickinson, H. G. (2006). Epigenetic asymmetry of imprinted genes in plant gametes. *Nat. Genet.* **38**, 876-878.
Henderson, I. R. and Jacobsen, S. E. (2008). Tandem repeats upstream of the Arabidopsis endogene SDC recruit non-CG DNA methylation and initiate siRNA spreading. *Genes Dev.* **22**, 1597-1606.
Herr, A. J., Jensen, M. B., Dalmay, T. and Baulcombe, D. C. (2005). RNA polymerase IV directs silencing of endogenous DNA. *Science* **308**, 118-120.
Hsieh, T. F., Shin, J., Uzawa, R., Silva, P., Cohen, S., Bauer, M. J., Hashimoto, M., Kirkbride, R. C., Harada, J. J., Zilberman, D. et al. (2011). Regulation of imprinted gene expression in Arabidopsis endosperm. *Proc. Natl. Acad. Sci. USA* **108**, 1755-1762.
Ikeda, Y., Kinoshita, Y., Susaki, D., Ikeda, Y., Iwano, M., Takayama, S., Higashiyama, T., Kakutani, T. and Kinoshita, T. (2011). HMG domain containing SSRP1 is required for DNA demethylation and genomic imprinting in Arabidopsis. *Dev. Cell* **21**, 589-596.
Ingouff, M., Sakata, T., Li, J., Sprunck, S., Dresselhaus, T. and Berger, F. (2009). The two male gametes share equal ability to fertilize the egg cell in Arabidopsis thaliana. *Curr. Biol.* **19**, R19-R20.
Jahnke, S. and Scholten, S. (2009). Epigenetic resetting of a gene imprinted in plant embryos. *Curr. Biol.* **19**, 1677-1681.
Jullien, P. E. and Berger, F. (2009). Gamete-specific epigenetic mechanisms shape genomic imprinting. *Curr. Opin. Plant Biol.* **12**, 637-642.
Jullien, P. E., Mosquna, A., Ingouff, M., Sakata, T., Ohad, N. and Berger, F. (2008). Retinoblastoma and its binding partner MSI1 control imprinting in Arabidopsis. *PLoS Biol.* **6**, e194.
Jullien, P. E., Susaki, D., Yelagandula, R., Higashiyama, T. and Berger, F. (2012). DNA methylation dynamics during sexual reproduction in Arabidopsis thaliana. *Curr. Biol.* **22**, 1825-1830.
Kaneko-Ishino, T. and Ishino, F. (2010). Retrotransposon silencing by DNA methylation contributed to the evolution of placentation and genomic imprinting in mammals. *Dev. Growth Differ.* **52**, 533-543.
Katz, A., Oliva, M., Mosquna, A., Hakim, O. and Ohad, N. (2004). FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J.* **37**, 707-719.
Kinoshita, T., Miura, A., Choi, Y., Kinoshita, Y., Cao, X., Jacobsen, S. E., Fischer, R. L. and Kakutani, T. (2004). One-way control of FWA imprinting in Arabidopsis endosperm by DNA methylation. *Science* **303**, 521-523.
Law, J. A. and Jacobsen, S. E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat. Rev. Genet.* **11**, 204-220.
Le, B. H., Cheng, C., Bui, A. Q., Wagmaister, J. A., Henry, K. F., Pelletier, J., Kwong, L., Belmonte, M., Kirkbride, R., Horvath, S. et al. (2010). Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors. *Proc. Natl. Acad. Sci. USA* **107**, 8063-8070.
Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H. and Ecker, J. R. (2008). Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell* **133**, 523-536.
Luo, M., Taylor, J. M., Spriggs, A., Zhang, H., Wu, X., Russell, S., Singh, M. and Koltunow, A. (2011). A genome-wide survey of imprinted genes in rice seeds reveals imprinting primarily occurs in the endosperm. *PLoS Genet.* **7**, e1002125.
Martienssen, R. A., Kloc, A., Slotkin, R. K. and Tanurdzić, M. (2008). Epigenetic inheritance and reprogramming in plants and fission yeast. *Cold Spring Harb. Symp. Quant. Biol.* **73**, 265-271.
McCole, R. B., Loughran, N. B., Chahal, M., Fernandes, L. P., Roberts, R. G., Fraternali, F., O'Connell, M. J. and Oakey, R. J. (2011). A case-by-case evolutionary analysis of four imprinted retrogenes. *Evolution* **65,** 1413-1427.
Mirouze, M., Reinders, Bucher, JE., Nishimura, T., Schneeberger, K., Ossowski, S., Cao, J., Weigel, D., Paszkowski, J. and Mathieu, O. (2009). Selective epigenetic control of retrotransposition in Arabidopsis. *Nature* **461**, 427-430.
Mosher, R. A., Schwach, F., Studholme, D. and Baulcombe, D. C. (2008). PolIVb influences RNA-directed DNA methylation independently of its role in siRNA biogenesis. *Proc. Natl. Acad. Sci. USA* **105**, 3145-3150.
Mosher, R. A., Melnyk, C. W., Kelly, K. A., Dunn, R. M., Studholme, D. J. and Baulcombe, D. C. (2009). Uniparental expression of PolIV-dependent siRNAs in developing endosperm of Arabidopsis. *Nature* **460**, 283-286.
Mosher, R. A., Tan, E. H., Shin, J., Fischer, R. L., Pikaard, C. S. and Baulcombe, D. C. (2011). An atypical epigenetic mechanism affects uniparental expression of Pol IV-dependent siRNAs. *PLoS ONE* **6**, e25756.
Numa, H., Kim, J. M., Matsui, A., Kurihara, Y., Morosawa, T., Ishida, J., Mochizuki, Y., Kimura, H., Shinozaki, K., Toyoda, T. et al. (2010). Transduction of RNA-directed DNA methylation signals to repressive histone marks in Arabidopsis thaliana. *EMBO J.* **29**, 352-362.
Okamura, K., Yamada, Y., Sakaki, Y. and Ito, T. (2004). An evolutionary scenario for genomic imprinting of Impact lying between nonimprinted neighbors. *DNA Res.* **11**, 381-390.
Onodera, Y., Haag, J. R., Ream, T., Costa Nunes, P., Pontes, O. and Pikaard, C. S. (2005). Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell* **120**, 613-622.
Pontier, D., Yahubyan, G., Vega, D., Bulski, A., Saez-Vasquez, J., Hakimi, M. A., Lerbs-Mache, S., Colot, V. and Lagrange, T. (2005). Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in Arabidopsis. *Genes Dev.* **19**, 2030-2040.
Saze, H. and Kakutani, T. (2007). Heritable epigenetic mutation of a transposon-flanked Arabidopsis gene due to lack of the chromatin-remodeling factor DDM1. *EMBO J.* **26**, 3641-3652.
Saze, H., Mittelsten Scheid, O. and Paszkowski, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nat. Genet.* **34**, 65-69.
Schmittgen, T. D. and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**, 1101-1108.
Schoft, V. K., Chumak, N., Choi, Y., Hannon, M., Garcia-Aguilar, M., Machlicova, A., Slusarz, L., Mosiolek, M., Park, J. S., Park, G. T. et al. (2011). Function of the DEMETER DNA glycosylase in the Arabidopsis thaliana male gametophyte. *Proc. Natl. Acad. Sci. USA* **108**, 8042-8047.
Tiwari, S., Schulz, R., Ikeda, Y., Dytham, L., Bravo, J., Mathers, L., Spielman, M., Guzmán, P., Oakey, R. J., Kinoshita, T. et al. (2008). MATERNALLY EXPRESSED PAB C-TERMINAL, a novel imprinted gene in Arabidopsis, encodes the conserved C-terminal domain of polyadenylate binding proteins. *Plant Cell* **20**, 2387-2398.
Walter, J. and Paulsen, M. (2003). The potential role of gene duplications in the evolution of imprinting mechanisms. *Hum. Mol. Genet.* **12** Suppl. 2, R215-R220.
Watanabe, T., Tomizawa, S., Mitsuya, K., Totoki, Y., Yamamoto, Y., Kuramochi-Miyagawa, S., Iida, N., Hoki, Y., Murphy, P. J., Toyoda, A. et al. (2011). Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse Rasgrf1 locus. *Science* **332**, 848-852.
Waters, A. J., Makarevitch, I., Eichten, S. R., Swanson-Wagner, R. A., Yeh, C. T., Xu, W., Schnable, P. S., Vaughn, M. W., Gehring, M. and Springer, N. M. (2011). Parent-of-origin effects on gene expression and DNA methylation in the maize endosperm. *Plant Cell* **23**, 4221-4233.
Wilkins, J. F. and Haig, D. (2003). What good is genomic imprinting: the function of parent-specific gene expression. *Nat. Rev. Genet.* **4**, 359-368.
Wolff, P., Weinhofer, I., Seguin, J., Roszak, P., Beisel, C., Donoghue, M. T., Spillane, C., Nordborg, M., Rehmsmeier, M. and Köhler, C. (2011). High-resolution analysis of parent-of-origin allelic expression in the Arabidopsis Endosperm. *PLoS Genet.* **7**, e1002126.
Zhang, M., Zhao, H., Xie, S., Chen, J., Xu, Y., Wang, K., Zhao, H., Guan, H., Hu, X., Jiao, Y. et al. (2011). Extensive, clustered parental imprinting of protein-coding and noncoding RNAs in developing maize endosperm. *Proc. Natl. Acad. Sci. USA* **108**, 20042-20047.