

Published in final edited form as:

*Plant J.* 2012 September ; 71(5): 776–786. doi:10.1111/j.1365-313X.2012.05028.x.

## Histone H1 affects gene imprinting and DNA methylation in *Arabidopsis*

Matthew Rea<sup>†</sup>, Wenguang Zheng<sup>†</sup>, Ming Chen<sup>‡</sup>, Christopher Braud, Drutdaman Bhangu, Tara N. Rognan, and Wenyan Xiao<sup>\*</sup>

Department of Biology, Saint Louis University, 3507 Laclede Ave, St. Louis, MO 63103, USA

### Summary

Imprinting, parent-of-origin expression of alleles, plays an important role in regulating development in mammals and plants. DNA methylation catalyzed by DNA methyltransferases plays a pivotal role in regulating imprinting by silencing parental alleles. DEMETER (DME), a DNA glycosylase functioning in the base-excision DNA repair pathway, can excise 5-methylcytosine from DNA and regulate genomic imprinting in *Arabidopsis*. DME demethylates the maternal *MEDEA* (*MEA*) promoter in endosperm, thus resulting in expression of the maternal *MEA* allele. However, it is not known whether DME interacts with other proteins in regulating gene imprinting. Here we report the identification of histone H1.2 as a DME-interacting protein in a yeast two-hybrid screen and confirmation of their interaction by the in vitro pull-down assay. Genetic analysis of the loss-of-function histone *h1* mutant shows that the maternal histone *H1* allele is required for DME regulation of the *MEA*, *FWA*, and *FIS2* imprinting in *Arabidopsis* endosperm while the paternal allele is dispensable. Furthermore, we show that mutations in histone *H1* result in an increase of DNA methylation in the maternal *MEA* and *FWA* promoter in endosperm. Our results suggest that histone H1 is involved in the DME-mediated DNA methylation and gene regulation at imprinted loci.

### Keywords

gene imprinting; DEMETER; histone H1; endosperm; DNA methylation; DNA demethylation; epigenetics

### INTRODUCTION

Imprinting, the preferential expression of either maternal or paternal alleles, is an epigenetic phenomenon in plants and mammals (Reik and Walter, 2001; Feil and Berger, 2007; Huh *et al.*, 2008). One of the widely accepted hypotheses of genomic imprinting is the parental conflict theory, which states that female and male parents have different interests in fitness of their progeny when one female can mate with multiple males (Ohad, 2007). The female parent wants all offspring to survive and thus prefers expression of genes that allocate limited resources equally to all offspring, whereas a male is only interested in its progeny, therefore favoring expression of genes that preferentially allocate resources to his offspring.

<sup>\*</sup>For correspondence: Fax, 314-977-3658; Tel, 314-977-2547; wxiao@slu.edu.

<sup>†</sup>These authors contributed equally to this work

<sup>‡</sup>Present address: National Key Laboratory for Crop Genetic Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Thus, different interests of female and male parents result in parent-of-origin expression of parental alleles of imprinted genes. DNA methylation, an addition of a methyl group at the 5-carbon position of cytosine, is an important epigenetic mechanism for regulating transposon silencing, gene expression, imprinting, and diseases (Feng *et al.*, 2010; Law and Jacobsen, 2010). DNA methylation was thought to be permanent and unchanging after the methyl group is added by DNA methyltransferases. However, evidence has shown that DNA methylation is a dynamic process and the 5-methylcytosine can be actively excised by the DNA glycosylases, DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), or DME-LIKE 2 (DML2), and replaced with a cytosine through the Base Excision Repair Pathway in *Arabidopsis* (Agius *et al.*, 2006; Gehring *et al.*, 2006; Huh *et al.*, 2008; Niehrs, 2009). It had been debated whether 5-methylcytosine DNA glycosylases exist in mammals. Very recently, it has been reported that the DNA repair enzyme thymine DNA glycosylase can perform active demethylation by deamination-base excision repair in mammals (Cortellino *et al.*, 2011).

Differing from mammals, there is haploid gametophytic growth in plants, i.e. postmeiotic cell divisions before fertilization. In *Arabidopsis*, a haploid megaspore at the end of meiosis undergoes three mitotic divisions to form a seven-cell, eight-nucleus female gametophyte containing the egg, central, synergid, and antipodal cells; the fusion of two haploid nuclei makes the nucleus of the diploid central cell. In the male gametophyte, a haploid microspore at the end of meiosis undergoes a mitotic cell division to give rise to one vegetative and one generative nucleus, and then the generative cell undergoes a mitotic cell division to result in two haploid sperm cells. There is also a double fertilization event that underlies gene imprinting in flowering plants. Fertilization of the egg cell by one sperm cell gives rise to a diploid embryo that ultimately generates the organs, tissues, and meristems of the plant (Goldberg *et al.*, 1994). Fertilization of the central cell by the second sperm generates the triploid endosperm that supports embryo or seedling growth (Brown *et al.*, 1999; Gehring *et al.*, 2004). Imprinting mainly occurs in the endosperm tissue in plants (Hsieh *et al.*, 2011), whereas imprinting can occur in embryos and in different adult tissues in mammals (Constancia *et al.*, 2004; Gregg *et al.*, 2010b).

*MEDEA* (*MEA*), a SET domain Polycomb group gene, was the first plant gene shown to be imprinted (maternally expressed and paternally silenced) in endosperm (Kinoshita *et al.*, 1999), and its maternal expression is controlled by DNA methylation and demethylation (Xiao *et al.*, 2003; Gehring *et al.*, 2006; Jullien *et al.*, 2006a). DNA METHYLTRANSFERASE 1 (*MET1*) is responsible for maintaining CpG methylation at the *MEA* locus (Xiao *et al.*, 2003), whereas DME excises 5-methylcytosine of the maternal *MEA* allele, thus activating its expression (Gehring *et al.*, 2006; Jullien *et al.*, 2006b). Interestingly, silencing of the paternal *MEA* allele is not controlled by DNA methylation. Rather, the maternally expressed Polycomb group proteins, including *MEA*, maintain the paternal *MEA* allele silencing (Gehring *et al.*, 2006). *FLOWERING WAGENINGEN* (*FWA*) homeodomain leucine zipper transcription factor and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) are also shown as imprinted genes in endosperm and regulated by DME (Kinoshita *et al.*, 2004; Jullien *et al.*, 2006a). Recently, it has been shown that *STRUCTURE SPECIFIC RECOGNITION PROTEIN 1* (*SSRPI*), a high mobility group (HMG) domain containing component of a FACILITATES CHROMATIN TRANSCRIPTION/TRANSACTION (FACT) histone chaperone complex, is required for DNA demethylation and regulation of *MEA* imprinted genes, suggesting chromatin modification and DNA demethylation are linked in epigenetic reprogramming in *Arabidopsis* (Ikeda *et al.*, 2011).

Chromatin, consisting of DNA and histone proteins, is the physiological template of genetic information in eukaryotes. Usually two molecules of each H2A, H2B, H3 and H4 form an

octamer of nucleosome core and approximately two superhelical turns of DNA wrap around it to form a nucleosome (Jenuwein and Allis, 2001). Histone H1, a linker histone found between nucleosomes, is essential for organizing nucleosomes into the 30-nm chromatin by binding to the interior of the filament (Thoma and Koller, 1977; Graziano *et al.*, 1994). Histone H1 has three domains in most species: a central globular domain, an N-terminal, and C-terminal tails (Hartman *et al.*, 1977). Eukaryotic cells normally contain similar molar amounts of linker histone H1 and nucleosomes (Fan *et al.*, 2003), i.e., nearly one H1 molecule for each core particle. Genetic evidence has shown that reduced amounts of H1 in cells result in shortening of the space between nucleosomes, altered chromatin structures, and changes in gene expression (Fan *et al.*, 2003). Recent studies have shown that H1 cannot be simply defined as a global transcriptional repressor since histone H1 is also involved in transcriptional activation (Fan *et al.*, 2005; Hashimoto *et al.*, 2010). Reducing histone H1 can affect DNA methylation patterns of some imprinted genes in mammals and plants (Fan *et al.*, 2005; Wierzbicki and Jerzmanowski, 2005). In *Arabidopsis*, it has been reported that 13 genes encode proteins containing domains similar to the conserved histone H1 globular domain, but only three genes *AT1G06760*, *AT2G30620*, and *AT2G18050* were shown as linker histones through molecular and biochemical analysis, and were named as *H1-1*, *H1-2*, and *H1-3*, respectively (Gantt and Lenvik, 1991; Ascenzi and Gantt, 1999a; Fan *et al.*, 2005; Wierzbicki and Jerzmanowski, 2005). Other *H1*-like genes are HMG and MYB domain-like DNA-binding proteins (Gantt and Lenvik, 1991; Ascenzi and Gantt, 1999a). *H1-1* and *H1-2* have 85% sequence identity at the nucleotide level in the globular domain while *H1-3* shares less homology and can be induced by dehydration and abscisic acid (Ascenzi and Gantt, 1997, 1999b).

In this study, we performed a yeast two-hybrid screen and identified histone H1.2 (previously called H1-2) as a DME-interacting protein. We used a genetic approach to investigate the role of histone H1 in modulating gene imprinting and found that the maternal histone *H1* allele is required for DME regulation of the imprinted expression of *MEA*, *FWA* and *FIS2* while the paternal allele is dispensable in *Arabidopsis* endosperm. Our result also indicates that mutations in histone *H1* result in an increase of DNA methylation in the maternal *MEA* and *FWA* promoter in endosperm, which suggests that histone H1 is involved in the DME-mediated DNA demethylation and gene expression at imprinted loci in *Arabidopsis*.

## RESULTS

### Identification of the histone H1 as a DME-interacting protein

DME, a DNA glycosylase that contains a conserved helix-hairpin-helix domain, can excise 5-methylcytosine residues at the imprinted *MEA* promoter in the *Arabidopsis* endosperm (Gehring *et al.*, 2006). Are there other components involved in the DME-mediated DNA demethylation process? We hypothesized that there was a scaffold protein that interacted with DME and affected DNA demethylation at imprinted loci. To identify such a protein, we performed a yeast two-hybrid screen using the full-length DME as a bait. We screened cDNA libraries from *Arabidopsis* flowers as described (Fan *et al.*, 1997; Wang *et al.*, 2006). Approximately 179,000 clones were screened from the yeast two-hybrid cDNA libraries and 17 positive colonies were obtained. One of sequenced clones was identified as histone *H1.2* (*AT2G30620*, called *H1-2* in previous references). As shown in Figure 1a, H1.2 interacted with DME in yeast and activated the reporter genes *His3* and *LacZ*. The quantitative assay showed that  $\beta$  – galactosidase activity in the yeast with constructs *DME-pAS1* and *H1.2-pACT* (1.67 units) was 42-fold higher than that in the control yeast (0.04 units) (Figure 1a). To confirm the interaction between H1.2 and DME, an *in vitro* pull-down assay was performed. We fused H1.2 with Glutathione-S-Transferase (GST) epitope tag, expressed the fusion protein in bacteria, and purified it. When the fused maltose binding protein DME

(MBP-DME) was purified and used as an input through GST-H1.2 bound column, the result showed that the fusion protein MBP-DME could be pulled down by GST-H1.2, but could not be pulled down by the control GST (Figure 1b). When MBP was used as an input in another control experiment, it could not be pulled down by either the fusion protein GST-H1.2 or GST itself (Figure 1b). These experiments demonstrate that H1.2 interacts with DME.

### The histone *H1* gene family in *Arabidopsis*

Histone H1 is a linker histone functioning in chromatin folding (Graziano *et al.*, 1994; Bustin *et al.*, 2005). It has been shown that there are several histone H1 variants in mammals (Fan *et al.*, 2001; Wierzbicki and Jerzmanowski, 2005; Hashimoto *et al.*, 2010). In *Arabidopsis*, there are three *H1* linker histone genes (*AT1G06760* [*H1.1*], *AT2G30620* [*H1.2*], and *AT2G18050* [*H1.3*]) (Gantt and Lenvik, 1991; Ascenzi and Gantt, 1999a; Fan *et al.*, 2005; Wierzbicki and Jerzmanowski, 2005). To test if H1.1 and H1.3 also interact with DME, we cloned the *H1.1* and *H1.3* with *Gal4* DNA activation domain (*H1.1-pACT(AD)* and *H1.3-pACT(AD)*, respectively) and examined their interaction with *DME* fused with *Gal4* DNA binding domain in *pAS1 (DME-pAS1(DB))* in a yeast two-hybrid assay, the result showed that both H1.1 and H1.3 interact with DME, and activated the reporter genes *His3* and *LacZ* in yeast (Figure 1c). The quantitative assay of  $\beta$ -galactosidase activity also indicated that H1.1 and H1.3 interact with DME in the yeast two-hybrid assay (Figure 1c). When yeast culture was serially diluted and spotted onto selection dropout medium, the result showed that H1.2, H1.1 and H1.3 interacted with DME (Figure S1).

The transgenic RNAi line to silence histone *H1.1*, *H1.2*, and *H1.3* was reported (Wierzbicki and Jerzmanowski, 2005). Since the RNAi approach using the transgene is dominant and cannot be used for imprinting studies, we searched for recessive T-DNA insertional mutants of the histone *H1* genes in the *Arabidopsis* Biological Resource Center database. By genotyping T-DNA insertional lines (Figure 2a), we found *h1.1-1* (Salk\_128430C) has a T-DNA insertion in the first exon (215 bp downstream of transcription start), and *h1.2-1* (Salk\_002142) and *h1.3-1* (Salk\_025209) have a T-DNA insertion in the promoter (382 bp and 62 bp upstream of transcription start, respectively). To determine if the putative mutants are knockout or knockdown alleles, we examined expression of histone *H1.1*, *H1.2*, and *H1.3* in their respective mutant endosperm tissues by RT-PCR. The results show that *h1.1-1* is a knockout allele and *h1.2-1* and *h1.3-1* are knockdown alleles (Figure 2b).

### Mutations in histone *H1* affect the *MEA*, *FWA* and *FIS2* imprinting

It has been shown that histone *H1* depletion in mouse embryonic stem cells affects expression of imprinted genes (Fan *et al.*, 2005). Since histone H1 interacts with DME (Figure 1) and DME is required for the maternal *MEA* allele expression in endosperm (Choi *et al.*, 2002), we speculated that mutations in histone *H1* might affect the *MEA* imprinting in *Arabidopsis*. To test this hypothesis, we utilized the polymorphism of the *MEA* locus between ecotypes Col-0 and RLD, performed reciprocal genetic crosses between the single mutant *h1.2-1*, double mutant *h1.1-1 h1.2-1* and *h1.2-1 h1.3-1*, and triple mutant *h1.1-1 h1.2-1 h1.3-1* in Col-0 background with RLD plants, and examined allele specific expression of *MEA* in the crossed silique endosperm (Figure 3). In the self-pollinated single mutant *h1.2-1* (*h1.2-1* X *h1.2-1*), expression of the maternal *MEA* allele was reduced compared with that in the control crosses (Col X Col and Col X RLD), and *MEA* expression is further reduced in the self-pollinated double mutants (*h1.1-1 h1.2-1* X *h1.1-1 h1.2-1* and *h1.2-1 h1.3-1* X *h1.2-1 h1.3-1*) and undetectable in the triple mutant (*h1.1-1 h1.2-1 h1.3-1* X *h1.1-1 h1.2-1 h1.3-1*) (Figure 3a). When the maternal histone *h1* single, double and triple mutants were crossed with wild type RLD male (Figure 3b), we obtained a similar result as that in self-pollinated mutants: the maternal *MEA* expression was reduced in the *h1.2-1*

cross (*h1.2-1* X RLD), was again reduced in the double mutant crossed with RLD (*h1.1-1 h1.2-1* X RLD and *h1.2-1 h1.3-1* X RLD), and undetectable in the triple mutant cross (*h1.1-1 h1.2-1 h1.3-1* X RLD). Interestingly, when the histone *h1* single, double and triple mutants as the male parent were crossed with wild type RLD female (Figure 3c), the maternal *MEA* allele from RLD ecotype was expressed to a similar level as the wild type control cross (RLD X RLD or RLD X Col). This suggests that the histone *h1* mutations from the male parent did not affect expression of the maternal *MEA* allele in the crosses. Together, these results indicate that mutations in the histone *H1* affect expression of the imprinted *MEA* gene in *Arabidopsis*.

DME also regulates imprinted expression of *FWA* and *FIS2* in endosperm (maternally expressed and paternally silenced) (Kinoshita *et al.*, 2004; Jullien *et al.*, 2006a). To examine whether mutations in *H1* affects imprinting of *FWA* and *FIS2*, we used RT-PCR to measure allele specific expression of *FWA* and *FIS2* in endosperm of the reciprocal crosses between the triple mutant *h1.1-1 h1.2-1 h1.3-1* in Col-0 and wild type *Ler*. The result showed that expression of the maternal *FWA* and *FIS2* alleles were significantly reduced when the triple mutant female was crossed with wild type male, whereas expression of the maternal alleles was not affected in the reciprocal cross with the triple mutant *h1.1-1 h1.2-1 h1.3-1* as male (Figure 4). This result suggests that histone *H1* might have a broad effect on expression of imprinted genes in *Arabidopsis*.

### Effects of the histone *H1* mutation on methylation of the maternal *MEA* and *FWA* promoter in endosperm

It has been reported that the maternal *MEA* promoter, as well as the 3' repeats, are methylated and its expression is antagonistically controlled by MET1 and DME (Xiao *et al.*, 2003; Gehring *et al.*, 2006). In the *Arabidopsis* RNAi lines with reduced expression of *H1*, there is a significant increase in CpG methylation in the 183-bp intergenic sequence repeats adjacent to the imprinted *MEA* gene (*MEA-ISR*) in leaf tissues (Wierzbicki and Jerzmanowski, 2005). We have shown that the histone H1 interacts with the DME protein *in vitro* (Figure 1). If H1 is required for the DME-mediated DNA demethylation, mutations in *H1* would result in an increase in DNA methylation in the maternal *MEA* promoter in endosperm. To test this hypothesis, we isolated endosperm tissues from the F1 seed of the histone *h1* triple mutant (*h1.1-1 h1.2-1 h1.3-1*) female crossed with wild type RLD male as well as the endosperm of the control cross (Col-0 female X RLD male), extracted endosperm genomic DNA, and performed bisulfite sequencing of the – 500 bp *MEA* promoter, which is important for regulating *MEA* expression (Choi *et al.*, 2002; Choi *et al.*, 2004; Gehring *et al.*, 2006). The result showed that there was 12% methylated cytosine in the maternal *MEA* promoter in – 500 bp region of the control cross endosperm (Col-0 female X RLD male) (Figure 5a). However, there was 64% methylated cytosine in the maternal *MEA* promoter in – 500 bp region in the endosperm of the histone *h1* triple mutant (*h1.1-1 h1.2-1 h1.3-1*) female crossed with wild type RLD male (Figure 5a), a significant increase in DNA methylation. For the paternal *MEA* allele, the DNA methylation levels were similar in wild type control and the triple mutant cross. This result shows that mutations in histone *H1* from the maternal parent cause an increase in DNA methylation of the maternal *MEA* promoter in the endosperm, which might in turn suppress the maternal *MEA* expression in the endosperm.

*FWA* is imprinted and regulated by DME demethylation in *Arabidopsis* endosperm (Kinoshita *et al.*, 2004). Since the *H1* mutation affected *FWA* imprinting (Figure 4a), we speculated that the *H1* mutation could have an effect on methylation of the 5' direct repeats of *FWA* (Soppe *et al.*, 2000). To test this, we examined methylation status of the 5' repeats of *FWA* in the F1 endosperm of the mutant and wild type crosses. The result showed that there was 23% methylated CpG, 16% CpHpG, and 9% CpHpH in the maternal 5' direct



repeats of *FWA* in the control cross endosperm (Col-0 female X *Ler* male) (Figure 5b). However, there was a substantial increase in DNA methylation: 55% methylated CpG, 22% CpHpG, and 11% CpHpH in the maternal 5' repeat region in the endosperm of the histone *h1* triple mutant (*h1.1-1 h1.2-1 h1.3-1*) female crossed with wild type *Ler* male (Figure 5b). For the paternal *FWA* allele, the DNA methylation levels were similar in the triple mutant cross compared with wild type control (Figure 5b). This result indicated that mutations in histone *H1* from the maternal parent result in an increase in CpG methylation of the maternal 5' repeats of *FWA* in the endosperm.

### Effects of the histone *h1* mutation on plant growth and development

The self-pollinated heterozygous *MEA/mea* mutant plant gives rise to approximately 50% aborted seed and the homozygous *mea/mea* is usually not viable (Kinoshita *et al.*, 1999; Vielle-Calzada *et al.*, 1999; Luo *et al.*, 2000). If the *H1* mutation completely abolishes the maternal *MEA* expression in the triple mutant *h1.1-1 h1.2-1 h1.3-1* plants, we would observe nearly 100% seed abortion in the self-pollinated triple mutant. However, we did not see the expected 100% seed abortion, but 16.7% seed abortion in the self-pollinated F<sub>3</sub> triple mutant *h1.1-1 h1.2-1 h1.3-1* plants (74 aborted seeds among total 444 seeds, Table 1). We observed 13.4% aborted seed (82 aborted seeds among total 611 seeds) when the F<sub>3</sub> triple *h1.1-1 h1.2-1 h1.3-1* mutant female was crossed with wild type Col-0 male, and 4.8% aborted seed (27/562) in the reciprocal cross (Table 1). It seemed that the seed abortion phenotype caused by the *h1* mutation was different from that in *mea* (i.e. endosperm over proliferation followed by seed collapse). The lack of *mea*-like seed abortion could be due to very low expression of *MEA* in the triple *h1.1-1 h1.2-1 h1.3-1* mutant. The reason might be that the *h1.2-1* and *h1.3-1* mutants are not null alleles although we did not detect the maternal *MEA* expression in the triple mutant under our RT-PCR conditions (Figure 3). In *Arabidopsis*, suppression of histone *H1* using RNAi approaches caused developmental abnormalities (Wierzbicki and Jerzmanowski, 2005). In the F<sub>1</sub> to F<sub>3</sub> progeny, we did not observe any developmental phenotype except some aborted seeds in the *h1* mutant. However, in the self-pollinated F<sub>4</sub> triple mutant *h1.1-1 h1.2-1 h1.3-1* progeny, we observed some mutant plants showing small stature (Figure 6a), delayed flowering (Figure 6b), and shorter siliques (Figure 6c). Since the abnormal developmental defects were observed in the self-pollinated F<sub>4</sub> generation and there was no genomic hypomethylation in the *h1* mutant (Figure S2), we speculate that sporadic developmental abnormalities can be due to epigenetic alteration in the *h1* mutant plants – an epiallele that is not associated with the *H1* locus.

### The histone *H1* is not an imprinted gene in *Arabidopsis*

Since only the maternal *H1* mutation affects expression of the maternal alleles of *MEA*, *FWA*, and *FIS2*, whereas the *H1* mutation from the paternal side has no effect (Figure 3), we were curious whether the parent-of-origin effect of *H1* on expression of *MEA*, *FWA*, and *FIS2* in endosperm was due to the imprinted feature of *H1* itself. To test this hypothesis, we crossed Col-0 with RLD, isolated embryo and endosperm RNA from crossed seed at 8 DAP, and designed a dCAPS marker to distinguish the parental *H1.2* allele. The result showed that the histone *H1.2* is not imprinted in either embryo or endosperm (Figure 7a).

We have shown that mutations in the maternal *H1* affect methylation status of the maternal *MEA* promoter and expression of the maternal *MEA* (Figure 3 and 4). We thought it was because *H1* is involved, directly or indirectly, in the DME-mediated DNA demethylation. To exclude the possibility that DME expression was affected by the *H1* mutation, we examined expression of *DME* in ovules of the triple mutant *h1.1-1 h1.2-1 h1.3-1* as well as wild type. The result showed that *DME* expression in the triple mutant *h1.1-1 h1.2-1 h1.3-1*

was similar to that in wild type (Figure 7b), suggesting that the *H1* mutation did not affect the DME activity.

## DISCUSSION

### Histone H1 affects gene imprinting

Genomic imprinting is an evolutionary conserved epigenetic phenomenon in mammals and plants (Feil and Berger, 2007). Through genomic approaches, many imprinted genes have recently been identified in *Arabidopsis* (Gehring *et al.*, 2011; Hsieh *et al.*, 2011; McKeown *et al.*, 2011; Wolff *et al.*, 2011), rice (Luo *et al.*, 2011), and mammals (Gregg *et al.*, 2010a; Gregg *et al.*, 2010b), but detailed molecular mechanisms regulating genome imprinting is still elusive. Our study shows that histones H1.1, H1.2, and H1.3 interact with DME *in vitro* (Figure 1). Loss-of-function mutations in histone *H1* result in a significant increase in DNA methylation of the maternal *MEA* and *FWA* promoter (Figure 5) and cause a reduction in the maternal *MEA* and *FWA* expression in endosperm (Figures 3 and 4). Interestingly, only the maternal *H1* mutation has effects on *MEA* and *FWA* imprinting whereas the *H1* mutation from the paternal side has no effect (Figures 3 and 4). We found that the histone *H1.2* is not imprinted in either embryo or endosperm (Figure 7a). Differing from mammals in gametogenesis, there is a haploid gametophytic growth stage: postmeiotic cell divisions before fertilization in the flowering plants. DME expression is restricted to the central cell in the female gametophyte, the progenitor of the endosperm (Choi *et al.*, 2002). DME is also expressed in the vegetative cell of pollen that is not involved in fertilization, but not in sperm cells (Schoft *et al.*). When the maternal *h1* mutant plant was crossed with a wild type male, it is likely that the *h1* mutation in the female gametophyte influences targeting of DME to the imprinted *MEA* locus, thus affecting maternal *MEA* allele expression. Since only the vegetative cell in pollen expresses DME and sperm cells involved in fertilization does not express DME, the paternal wild type allele cannot rescue the defect caused by the maternal *h1* mutation. The result that *h1* mutation from the male has no effect on *MEA* imprinting suggests that H1 expression and its interaction with DME in the female gametophyte is sufficient for sustaining the maternal *MEA* expression in the central cell and endosperm.

### Role of histone H1 in the DME-mediated DNA demethylation in the *Arabidopsis* genome

How is a DNA glycosylase like DME targeted to a specific locus or transposon to perform its demethylase function in the genome? Does the plant with the *h1* mutation fail to target DME into the *MEA* locus in the central cell and lead to hypermethylation of the maternal *MEA* promoter, thus resulting in reduced maternal *MEA* expression? It is likely that DME interacts with some scaffold proteins, like H1 variants, to facilitate its localization to the imprinted locus, *MEA*. One possibility is that mutations in histone *H1* can abolish DME targeting or reduce its efficiency, and result in an increase in DNA methylation in the *MEA* promoter (Figure 5a), thus leading to reduced *MEA* expression in endosperm (Figure 3). It has been reported that there is a significant increase of CpG methylation in the *MEA* intergenic subtelomeric repeat (*MEA-ISR*) region in leaf tissues of the *H1* RNAi plants (Wierzbicki and Jerzmanowski, 2005), which is conceptually consistent with our result (Figure 5a). How can histone H1 affect DNA methylation? Our results suggest histone H1 plays a direct or indirect role in the DME-mediated DNA demethylation. Little is known what is the exact role of histone H1 in the DME-mediated DNA demethylation in the genome, and we speculate the following possible mechanisms. First, histone H1 has basic lysine-rich regions that interact with DNA (Hendzel *et al.*, 2004; Fang *et al.*, 2011) and this might enhance DME binding to DNA *in vivo*. H1 variants have direct effects on chromatin structure (Bustin *et al.*, 2005; Fan *et al.*, 2005), which might allow DME and/or transcription factors to access the chromatin, thus activating *MEA* expression in the central cell. Second,

it has been reported that other proteins can replace or exclude a nucleosome-specific histone H1 variant and affect chromatin structure (Swindle and Engler, 1998; Ju *et al.*, 2006). It is possible that some histone H1 variants associated with the methylated locus like *MEA* or transposable element can bring DME to DNA through their interaction, and then DME can replace histone H1 variants and open up chromatin, thus demethylating the *MEA* locus and activating its expression. Third, in our original yeast two-hybrid screen for DME-interacting proteins (Figure 1 and as described in the result), we also uncovered other nuclear proteins including transcription factors and proteins with motifs mediating protein-protein or DNA-protein interactions, which can play a more direct role in targeting DME into a specific locus in the genome. Characterization of other DME-interacting proteins might reveal molecular mechanisms of how the DME DNA glycosylase performs its demethylation in the genome.

## EXPERIMENTAL PROCEDURES

### Plant materials and growth conditions

T-DNA lines were ordered from ABRC for each of the three *H1* genes, *H1.1*, *H1.2*, and *H1.3*. The T-DNA insertion in *h1.1-1* (SALK\_128430C) was detected in the first exon by amplifying a 493-bp PCR fragment with primers: *SALK-128430-2*, 5'-GAATAAACGAAATCGAATCTC-3' and *LBa1*, 5'-TGGTTCACGTAGTGGGCCATCG-3'. The T-DNA insertion in *h1.2-1* (SALK\_002142) was detected in the promoter 382-bp upstream of transcription start by amplifying a 633-bp PCR fragment with primers: *H1.2 promoter TDNA*, 5'-CGTATATTTTCAGTAGAGGAAAACCTTGCC-3' and *LBa1* (see above). The T-DNA insertion in *h1.3-1* (SALK\_025209) was detected in the promoter 62-bp upstream of transcription start by amplifying a 552-bp PCR fragment with primers: *H1.3 promoter F2*, 5'-CTCTGGTTTAAACTTTCTCCCATC-3 and *LBa1*. Once T-DNA insertions were found in a plant, PCR amplification was used to determine homozygosity by amplifying an endogenous gene fragment across the T-DNA insertion. A 930-bp endogenous fragment was amplified in *H1.1* with primers: 5'-CGGGATCCATGTCAGAGGTGGAAATAGAG-3' and 5'-CCGCTGGAGTCACTTCTTAACCCTAGAAGA-3'; A 2074-bp endogenous fragment was amplified in *H1.2* with primers: *H1.2 promoter TDNA* (see above) and 5'-GCGAGCTCTCACTTCTTAGCCTCCCTAG-3'; A 1405-bp endogenous fragment was amplified in *H1.3* with primers: 5'-GCCTCTCGGTAAATGTGGTAAA-3' and 5'-CGGGATCCCAAGCAGCGGAAGCTT-3'. Three homozygous single mutants *h1.1-1*, *h1.2-1*, and *h1.3-1* were backcrossed to wild type for three times and then used to create the double mutants (*h1.1-1 h1.2-1*, *h1.1-1 h1.3-1*, *H1.2-1 h1.3-1*) and the triple mutant (*h1.1-1 h1.2-1 h1.3-1*). *Arabidopsis* plants were grown in growth chambers or greenhouse under 16-hour light and 8-hour dark at 23 °C.

### Yeast two-hybrid screen and assay

The full-length *DME* was generated by PCR amplification, and fused with the GAL4 DNA binding domain (DB) in *pAS1* (Fan *et al.*, 1997) and *pGBKT7* (Wang *et al.*, 2006). *DME* - DB was used as a bait to screen cDNA libraries fused with the GAL4 DNA activation domain (AD) in *pACT* as described (Fan *et al.*, 1997; Wang *et al.*, 2006). *H1.2* was a positive clone identified in the above yeast two-hybrid library screening. For experiments of H1.1 and H1.3 interact with DME in yeast, *Arabidopsis H1.1* and *H1.3* were amplified by PCR and fused with the GAL4 activation domain in *pACT*, *H1.1-pACT(AD)* and *H1.3-pACT(AD)*. The constructs were then transformed into the yeast containing *DME-pAS1(DB)* and interaction clones were selected on yeast dropout media. The concentration of 3-AT used in the selection medium was 12.5 mM if not specified. To measure  $\beta$ -galactosidase activity, the qualitative assay using a filter lift and quantitative assay using liquid culture were as described (Ausubel, 2008).



## Protein expression and purification

The construct for DME protein expression, c2x-DMEΔN677 was provided by Dr. Huh (Mok *et al.*, 2010). Expression of DMEΔN677 was performed as described (Gehring *et al.*, 2006). For the expression of MBP protein, BL21 cells containing pMAL-c2x (NEB) were induced at 0.3 mM IPTG in LB medium at 37°C for 3 hours. MBP and MBP-tagged DMEΔN677 proteins were purified with amylose resin (NEB). To make the expression construct for histone H1.2, the coding region of H1.2 gene was amplified by PCR with primers H1.2Ex-F (5'-CGCGGATCCATGTCTATAGAGGAAGAAAACGTT-3') and H1.2Ex-R (5'-CGCCTCGAGTCACTTCTTAGCCTTCCTAGTCGA-3') and cloned into *pGEX 4T-1* expression vector. The resulting plasmid was transformed into BL21 cells and induced with 1 mM IPTG in LB medium at 24°C for 3 hours. GST and GST-H1.2 fusion proteins were purified with glutathione-superflow resin (Clontech).

## *In vitro* pull-down assay

GST or GST-tagged histone H1 proteins were immobilized on glutathione-superflow resin and used as bait. The purified MBP or MBP-DMEΔN677 proteins as input were added to the resin and incubated in TBS (25 mM Tris-HCl, 150 mM NaCl, pH7.2) on nutator at 4°C for 3 hours. After four times of wash with TBS, the beads were resuspended in TBS and protein loading buffer. The beads were boiled for 5 min and centrifuged at 12,000 rpm for 3 min. Input proteins and the supernatants were separated on 10% SDS-PAGE, blotted, and detected by antibodies to MBP or GST.

## Gene expression and imprinting analysis

For examining expression of histone *H1.1*, *H1.2*, and *H1.3* in the respective mutant plants, endosperm tissues from seed at 8-DAP of respective plants were isolated and total RNA was extracted. Gene expression was examined by using semi-quantitative RT-PCR using 28–30 PCR amplification cycles. Primer pairs for amplifying cDNA of *H1.1*, *H1.2* and *H1.3* were: H1.1-F1 and H1.1-R1; H1.2-Forward and H1.2-R1; H1.3-NcoI Y2H-pACT2 and H1.3-Y2H-R1 BamHI, respectively. The primers used for histone *H1* expression cover the entire coding region of the genes, which distinguished cDNA from any possible genomic DNA contamination in the assay. *ACTIN* (*ACT*) was used as a control for the experiment. For investigating effects of histone *H1* mutations on *MEA* imprinting in *Arabidopsis*, endosperm tissues from seed of respective crosses at 8-DAP were collected and total RNA was isolated. The *MEA* alleles from Col-0 and RLD were distinguished by a dCAPS marker and *MEA* expression was examined by using RT-PCR as described (Kinoshita *et al.*, 1999). Expression of *FWA* and *FIS2* was examined by using primers and RT-PCR as described (Kinoshita *et al.*, 2004; Jullien *et al.*, 2006a).

## dCAPS marker for *H1-2*

dCAPS marker was designed with the polymorphism PERL0376855 between Col-0 and *Ler* ecotypes. PCR products amplified by primers dCAPS-F (5'-GTTCCAACGACTGTTGACTCAGGAGCAGC-3') and dCAPS-R (5'-GTATTGACTAGATCCAGTTCTCTCCTTC-3') were digested with Pst I restriction enzyme and separated on 3% agarose gel. The PCR product from Col-0 contains the Pst I site but the *Ler* allele does not.

## Bisulfite sequencing

Four independent triple mutant *h1.1-1 h1.2-1 h1.3-1* plants were used in the genetic cross for the bisulfite sequencing assay. Endosperm tissues from seed of respective crosses (Col-0 X RLD and *h1.1-1 h1.2-1 h1.3-1* X RLD) at 8-DAP were isolated and genomic DNA was extracted as described (Rea *et al.*, 2011). Genomic DNA was then digested with Xho I and

Pst I followed by bisulfite treatment. The bisulfite conversion was performed using Methylcode Bisulfite Conversion Kit (Invitrogen). After conversion, the *MEA* promoter at -500 bp region was PCR-amplified by using primers *mea7520TFc* and *mea7933TRc* for the top strand, *mea7529F* and *mea7935R* for the bottom strand, PCR fragments were then cloned into pCR2.1-TOPO vector (Invitrogen) and positive colonies were selected for sequencing as described (Xiao *et al.*, 2003). The sequences from the maternal *MEA* allele from Col-0 background were differentiated from those from the paternal RLD allele based on the polymorphism in the region between Col-0 and RLD ecotypes (Gehring *et al.*, 2006). For bisulfite sequencing 5' repeats of *FWA*, bisulfite treatment was the same as described above, but two rounds of semi-nested PCR were used to amplified the fragment of 5' repeats. The first round PCR cycling condition was as described (Pappas *et al.*, 2009). 0.5 µl of first round PCR product was used as template for the second round PCR. The PCR cycling condition for second round PCR was 94°C for 1 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72 °C for 1 min; 72°C for 5 min. To amplifying the top strand of 5' repeats of *FWA*, primers used in the first round PCR were *FWA-top F* (5'-GGTTTTATATTAATATTAAGAGTTATGGGTYGAAGTTT-3') and *FWA-top R* (5'-CAAATACTTTACACATAAACRAAAAACAAACAAATCRAA-3') as described (Chan *et al.*, 2006), and primers for the second round were *FWA-top F* and *FWA-TR2* (5'-AACCAAAATCATTCTCTAAACAAAATRTAAAAAATC-3'). To amplifying the bottom strand of 5' repeats of *FWA*, primers *FWA-bottom F* (5'-TAAAGTATTTTATATATAAGYGAAAAATAGATAAATTGG-3') and *FWA-bottom R* (5'-AATTCTATACTAATATCAAAAAATTATAAACCCRAAACCC-3') were used for the first round PCR, and primers *FWA-BF1* (5'-TTCTATACTAATATCAAARARTTATRRRCCRAARCCC-3') and *FWA-BR2* (5'-TAAAYAAAATGTAAAAAATYTGATTTTTGGYTGA-3') were used for the second round PCR. The maternal *FWA* allele (Col-0 ecotype) and paternal *FWA* allele (Ler ecotype) in the cross were distinguished based on the DNA sequence polymorphisms (Soppe *et al.*, 2000).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors thank Robert L. Fischer, in whose laboratory the original yeast two-hybrid screen was performed and W.X. greatly appreciates R.L. Fischer's generosity of letting him to carry on the project and his valuable comments on the manuscript. We thank Eric Odle and Kaushal Patel for maintenance of *Arabidopsis* plants, thank the ABRC at The Ohio State University for providing seeds and libraries, Ramin Yadegary for providing the yeast two-hybrid cDNA library, Jin Hoe Huh for providing DME clone, Andrzej Jerzmanowski for providing the *H1.1 H1.2 H1.3* RNAi construct, and Daniel Zilberman for providing his putative *h1* mutant seed. This work was supported by National Institutes of Health grants 1R15GM086846-01 and 3R15GM086846-01S1 to W. X.

## REFERENCES

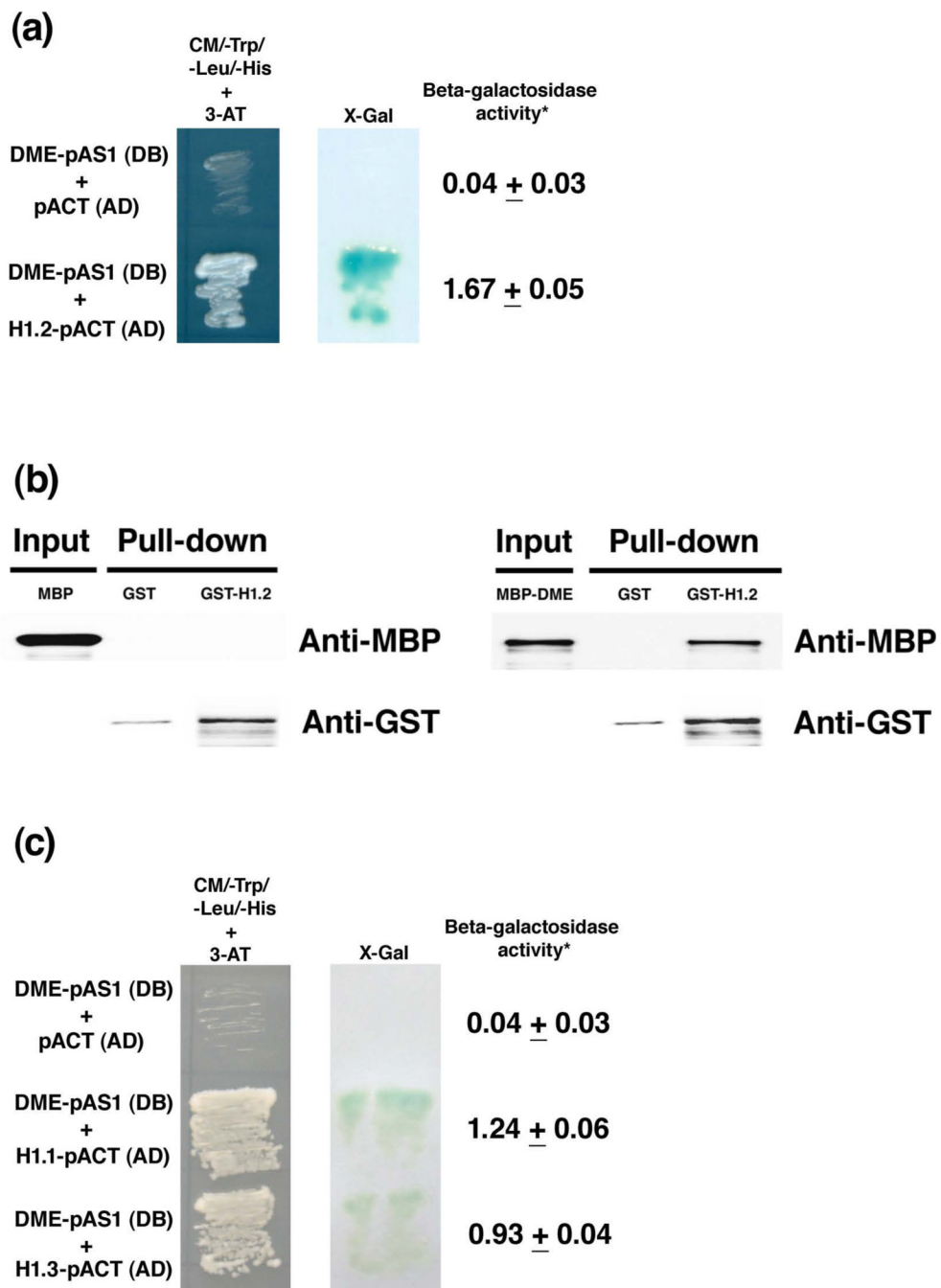
- Agius F, Kapoor A, Zhu JK. Role of the Arabidopsis DNA glycosylase/lyase ROS1 in active DNA demethylation. *Proc Natl Acad Sci U S A*. 2006; 103:11796–11801. [PubMed: 16864782]
- Ascenzi R, Gantt JS. A drought-stress-inducible histone gene in Arabidopsis thaliana is a member of a distinct class of plant linker histone variants. *Plant Mol Biol*. 1997; 34:629–641. [PubMed: 9247544]
- Ascenzi R, Gantt JS. Molecular genetic analysis of the drought-inducible linker histone variant in Arabidopsis thaliana. *Plant Mol Biol*. 1999a; 41:159–169. [PubMed: 10579484]
- Ascenzi R, Gantt JS. Subnuclear distribution of the entire complement of linker histone variants in Arabidopsis thaliana. *Chromosoma*. 1999b; 108:345–355. [PubMed: 10591994]

- Ausubel F, Brent R, Kingston R, Moore DD, Seidman JG, Smith JA, Struhl K. Current Protocols in Molecular Biology. 2008; 3:13.16.11–13.16.15. [PubMed: 18972385]
- Brown RC, Lemmon BE, Nguyen H, Olsen O-A. Development of endosperm in *Arabidopsis thaliana*. Sex. Plant Reprod. 1999; 12:32–42.
- Bustin M, Catez F, Lim JH. The dynamics of histone H1 function in chromatin. Mol Cell. 2005; 17:617–620. [PubMed: 15749012]
- Chan SW, Zhang X, Bernatavichute YV, Jacobsen SE. Two-step recruitment of RNA-directed DNA methylation to tandem repeats. PLoS Biol. 2006; 4:e363. [PubMed: 17105345]
- Choi Y, Harada JJ, Goldberg RB, Fischer RL. An invariant aspartic acid in the DNA glycosylase domain of DEMETER is necessary for transcriptional activation of the imprinted MEDEA gene. Proc Natl Acad Sci U S A. 2004; 101:7481–7486. [PubMed: 15128940]
- Choi Y, Gehring M, Johnson L, Hannon M, Harada JJ, Goldberg RB, Jacobsen SE, Fischer RL. DEMETER, a DNA Glycosylase Domain Protein, Is Required for Endosperm Gene Imprinting and Seed Viability in Arabidopsis. Cell. 2002; 110:33–42. [PubMed: 12150995]
- Constancia M, Kelsey G, Reik W. Resourceful imprinting. Nature. 2004; 432:53–57. [PubMed: 15525980]
- Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, Le Coz M, Devarajan K, Wessels A, Soprano D, Abramowitz LK, Bartolomei MS, Rambow F, Bassi MR, Bruno T, Fanciulli M, Renner C, Klein-Szanto AJ, Matsumoto Y, Kobi D, Davidson I, Alberti C, Larue L, Bellacosa A. Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. Cell. 2011; 146:67–79. [PubMed: 21722948]
- Fan HY, Hu Y, Tudor M, Ma H. Specific interactions between the K domains of AG and AGLs, members of the MADS domain family of DNA binding proteins. Plant J. 1997; 12:999–1010. [PubMed: 9418042]
- Fan Y, Sirotkin A, Russell RG, Ayala J, Skoultschi AI. Individual somatic H1 subtypes are dispensable for mouse development even in mice lacking the H1(0) replacement subtype. Mol Cell Biol. 2001; 21:7933–7943. [PubMed: 11689686]
- Fan Y, Nikitina T, Morin-Kensicki EM, Zhao J, Magnuson TR, Woodcock CL, Skoultschi AI. H1 linker histones are essential for mouse development and affect nucleosome spacing in vivo. Mol Cell Biol. 2003; 23:4559–4572. [PubMed: 12808097]
- Fan Y, Nikitina T, Zhao J, Fleury TJ, Bhattacharyya R, Bouhassira EE, Stein A, Woodcock CL, Skoultschi AI. Histone H1 depletion in mammals alters global chromatin structure but causes specific changes in gene regulation. Cell. 2005; 123:1199–1212. [PubMed: 16377562]
- Fang H, Clark DJ, Hayes JJ. DNA and nucleosomes direct distinct folding of a linker histone H1 C-terminal domain. Nucleic Acids Res. 2011
- Feil R, Berger F. Convergent evolution of genomic imprinting in plants and mammals. Trends Genet. 2007; 23:192–199. [PubMed: 17316885]
- Feng S, Jacobsen SE, Reik W. Epigenetic reprogramming in plant and animal development. Science. 2010; 330:622–627. [PubMed: 21030646]
- Gantt JS, Lenvik TR. Arabidopsis thaliana H1 histones. Analysis of two members of a small gene family. Eur J Biochem. 1991; 202:1029–1039. [PubMed: 1765064]
- Gehring M, Choi Y, Fischer RL. Imprinting and Seed Development. Plant Cell. 2004; 16
- Gehring M, Bubb KL, Henikoff S. Extensive demethylation of repetitive elements during seed development underlies gene imprinting. Science. 2009; 324:1447–1451. [PubMed: 19520961]
- Gehring M, Missirian V, Henikoff S. Genomic analysis of parent-of-origin allelic expression in Arabidopsis thaliana seeds. PLoS One. 2011; 6:e23687. [PubMed: 21858209]
- Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, Harada JJ, Goldberg RB, Fischer RL. DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. Cell. 2006; 124:495–506. [PubMed: 16469697]
- Goldberg RB, de Paiva G, Yadegari R. Plant embryogenesis: Zygote to seed. Science. 1994; 266:605–614. [PubMed: 17793455]
- Graziano V, Gerchman SE, Schneider DK, Ramakrishnan V. Histone H1 is located in the interior of the chromatin 30-nm filament. Nature. 1994; 368:351–354. [PubMed: 8127372]

- Gregg C, Zhang J, Butler JE, Haig D, Dulac C. Sex-specific parent-of-origin allelic expression in the mouse brain. *Science*. 2010a; 329:682–685. [PubMed: 20616234]
- Gregg C, Zhang J, Weissbourd B, Luo S, Schroth GP, Haig D, Dulac C. High-resolution analysis of parent-of-origin allelic expression in the mouse brain. *Science*. 2010b; 329:643–648. [PubMed: 20616232]
- Haig D, Westoby M. Parent-specific gene expression and the triploid endosperm. *American Naturalist*. 1989; 134:147–155.
- Haig D, Westoby M. Genomic imprinting in endosperm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Phil. Trans. R. Soc. Lond. B*. 1991; 333:1–14.
- Hartman PG, Chapman GE, Moss T, Bradbury EM. Studies on the role and mode of operation of the very-lysine-rich histone H1 in eukaryote chromatin. The three structural regions of the histone H1 molecule. *Eur J Biochem*. 1977; 77:45–51. [PubMed: 908338]
- Hashimoto H, Takami Y, Sonoda E, Iwasaki T, Iwano H, Tachibana M, Takeda S, Nakayama T, Kimura H, Shinkai Y. Histone H1 null vertebrate cells exhibit altered nucleosome architecture. *Nucleic Acids Res*. 2010; 38:3533–3545. [PubMed: 20156997]
- Hendzel MJ, Lever MA, Crawford E, Th'ng JP. The C-terminal domain is the primary determinant of histone H1 binding to chromatin in vivo. *J Biol Chem*. 2004; 279:20028–20034. [PubMed: 14985337]
- Hsieh TF, Shin J, Uzawa R, Silva P, Cohen S, Bauer MJ, Hashimoto M, Kirkbride RC, Harada JJ, Zilberman D, Fischer RL. Regulation of imprinted gene expression in Arabidopsis endosperm. *Proc Natl Acad Sci U S A*. 2011; 108:1755–1762. [PubMed: 21257907]
- Huh JH, Bauer MJ, Hsieh TF, Fischer RL. Cellular programming of plant gene imprinting. *Cell*. 2008; 132:735–744. [PubMed: 18329361]
- Jenuwein T, Allis CD. Translating the histone code. *Science*. 2001; 293:1074–1080. [PubMed: 11498575]
- Ikeda Y, Kinoshita Y, Susaki D, Iwano M, Takayama S, Higashiyama T, Kakutani T, Kinoshita T. HMG domain containing SSRP1 is required for DNA demethylation and genomic imprinting in Arabidopsis. *Dev Cell*. 2011; 21:589–596. [PubMed: 21920319]
- Ju BG, Lunyak VV, Perissi V, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MG. A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription. *Science*. 2006; 312:1798–1802. [PubMed: 16794079]
- Jullien PE, Kinoshita T, Ohad N, Berger F. Maintenance of DNA methylation during the Arabidopsis life cycle is essential for parental imprinting. *Plant Cell*. 2006a; 18:1360–1372. [PubMed: 16648367]
- Jullien PE, Katz A, Oliva M, Ohad N, Berger F. Polycomb group complexes self-regulate imprinting of the Polycomb group gene MEDEA in Arabidopsis. *Curr Biol*. 2006b; 16:486–492. [PubMed: 16527743]
- Kankel MW, Ramsey DE, Stokes TL, Flowers SK, Haag JR, Jeddelloh JA, Riddle NC, Verbsky ML, Richards EJ. Arabidopsis MET1 Cytosine Methyltransferase Mutants. *Genetics*. 2003; 163:1109–1122. [PubMed: 12663548]
- Kinoshita T, Yadegari R, Harada JJ, Goldberg RB, Fischer RL. Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm. *Plant Cell*. 1999; 11:1945–1952. [PubMed: 10521524]
- Kinoshita T, Miura A, Choi Y, Kinoshita Y, Cao X, Jacobsen SE, Fischer RL, Kakutani T. One-way control of FWA imprinting in Arabidopsis endosperm by DNA methylation. *Science*. 2004; 303:521–523. Published online 510.1126/science.1089835. [PubMed: 14631047]
- Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet*. 2010; 11:204–220. [PubMed: 20142834]
- Luo M, Bilodeau P, Dennis ES, Peacock WJ, Chaudhury A. Expression and parent-of-origin effects for FIS2, MEA, and FIE in the endosperm and embryo of developing Arabidopsis seeds. *Proc Natl Acad Sci U S A*. 2000; 97:10637–10642. [PubMed: 10962025]
- Luo M, Taylor JM, Spriggs A, Zhang H, Wu X, Russell S, Singh M, Koltunow A. A genome-wide survey of imprinted genes in rice seeds reveals imprinting primarily occurs in the endosperm. *PLoS Genet*. 2011; 7 e1002125.

- McKeown PC, Laouielle-Duprat S, Prins P, Wolff P, Schmid MW, Donoghue MT, Fort A, Duszynska D, Comte A, Lao NT, Wennblom TJ, Smant G, Kohler C, Grossniklaus U, Spillane C. Identification of imprinted genes subject to parent-of-origin specific expression in *Arabidopsis thaliana* seeds. *BMC Plant Biol.* 2011; 11:113. [PubMed: 21838868]
- Mok YG, Uzawa R, Lee J, Weiner GM, Eichman BF, Fischer RL, Huh JH. Domain structure of the DEMETER 5-methylcytosine DNA glycosylase. *Proc Natl Acad Sci U S A.* 2010; 107:19225–19230. [PubMed: 20974931]
- Moore T, Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends in Genetics.* 1991; 7:45–48. [PubMed: 2035190]
- Niehrs C. Active DNA demethylation and DNA repair. *Differentiation.* 2009; 77:1–11. [PubMed: 19281759]
- Ohad N. Plant development: parental conflict overcome. *Nature.* 2007; 447:275–276. [PubMed: 17507973]
- Pappas JJ, Toulouse A, Bradley WE. A modified protocol for bisulfite genomic sequencing of difficult samples. *Biol Proced Online.* 2009; 11:99–112. [PubMed: 19551458]
- Rea M, Chen M, Luan S, Bhangu D, Braud M, Xiao W. Determination of DNA methylation of imprinted genes in *Arabidopsis* endosperm. *J Vis Exp.* 2011 <http://www.jove.com/index/Details.stp?ID=2327>.
- Reik W, Walter J. Genomic imprinting: Parental influence on the genome. *Nature Rev. Genet.* 2001; 2:21–32. [PubMed: 11253064]
- Schoft VK, Chumak N, Choi Y, Hannon M, Garcia-Aguilar M, Machlicova A, Slusarz L, Mosiolek M, Park JS, Park GT, Fischer RL, Tamaru H. Function of the DEMETER DNA glycosylase in the *Arabidopsis thaliana* male gametophyte. *Proc Natl Acad Sci U S A.* 2011; 108:8042–8047. [PubMed: 21518889]
- Soppe WJJ, Jacobsen SE, Alonso-Blanco C, Jackson JP, Kakutani T, Koornneef M, Peeters AJM. The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol Cell.* 2000; 6:791–802. [PubMed: 11090618]
- Swindle CS, Engler JA. Association of the human papillomavirus type 11 E1 protein with histone H1. *J Virol.* 1998; 72:1994–2001. [PubMed: 9499053]
- Thoma F, Koller T. Influence of histone H1 on chromatin structure. *Cell.* 1977; 12:101–107. [PubMed: 561660]
- Thomas JO. The higher order structure of chromatin and histone H1. *J Cell Sci Suppl.* 1984; 1:1–20. [PubMed: 6397467]
- Vielle-Calzada JP, Thomas J, Spillane C, Coluccio A, Hoepfner MA, Grossniklaus U. Maintenance of genomic imprinting at the *Arabidopsis* *medea* locus requires zygotic DDM1 activity. *Genes Dev.* 1999; 13:2971–2982. [PubMed: 10580004]
- Wang D, Tyson MD, Jackson SS, Yadegari R. Partially redundant functions of two SET-domain polycomb-group proteins in controlling initiation of seed development in *Arabidopsis*. *Proc Natl Acad Sci U S A.* 2006; 103:13244–13249. [PubMed: 16924116]
- Wierzbicki AT, Jerzmanowski A. Suppression of histone H1 genes in *Arabidopsis* results in heritable developmental defects and stochastic changes in DNA methylation. *Genetics.* 2005; 169:997–1008. [PubMed: 15489532]
- Wolff P, Weinhofer I, Seguin J, Roszak P, Beisel C, Donoghue MT, Spillane C, Nordborg M, Rehmsmeier M, Kohler C. High-Resolution Analysis of Parent-of-Origin Allelic Expression in the *Arabidopsis* Endosperm. *PLoS Genet.* 2011; 7 e1002126.
- Xiao W, Gehring M, Choi Y, Margossian L, Pu H, Harada JJ, Goldberg RB, Pennell RI, Fischer RL. Imprinting of the MEA Polycomb gene is controlled by antagonism between MET1 methyltransferase and DME glycosylase. *Developmental Cell.* 2003; 5:891–901. [PubMed: 14667411]



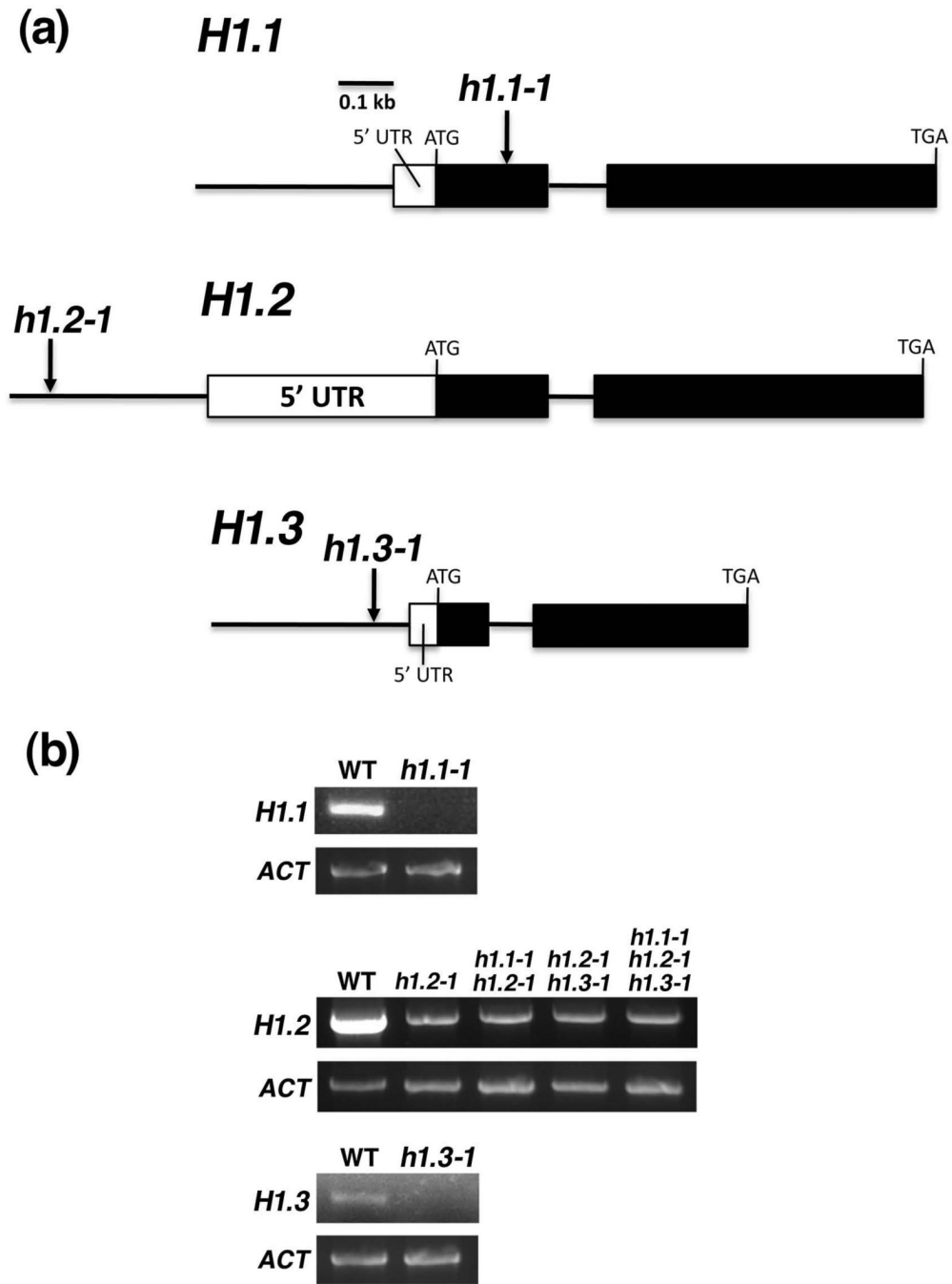


**Figure 1. Histone H1 proteins interact with DME**

(a) Histone H1.2 interacts with DME in yeast. Histone *H1.2* was isolated as a positive clone in a yeast two-hybrid screening of *Arabidopsis* cDNA library by using DME as a bait. Full-length DME fused with the GAL4 DNA binding domain, DME-pAS1 (DB), interacts with H1.2 fused with the GAL4 activation domain, H1.2-pACT (AD) (Fan *et al.*, 1997). The concentration for 3-AT was 12.5 mM. The X-Gal qualitative and quantitative assays were as described (Ausubel, 2008). \*The unit for galactosidase activity in the quantitative assay was  $1000 \times (A_{420} - 1.75 \times A_{550}) / (t \text{ (in minutes)} \times v \text{ (volume in ml)} \times OD_{600})$  in Figures 1a and 1c.

(b) H1.2 interacts with DME *in vitro*. GST or GST-H1.2 were immobilized on glutathione-superflow resin and used as bait to pull down MBP or MBP-DME. Proteins bound to the resin were separated by SDS-PAGE, blotted and probed with antibodies to MBP or GST. A partial DME with the N-terminal deletion (DME $\Delta$ N677) was used for the fusion protein (MBP-DME) as described (Gehring *et al.*, 2006).

(c) H1.1 and H1.3 interact with DME in yeast. Full-length DME fused with the DNA binding domain, DME-pAS1 (DB), interacts with H1.1 and H1.3 fused with the GAL4 activation domain, H1.1-pACT(AD) and H1.3-pACT (AD), respectively. The concentration for 3-AT was 12.5 mM. The X-Gal assay was as described above in Figure 1a.

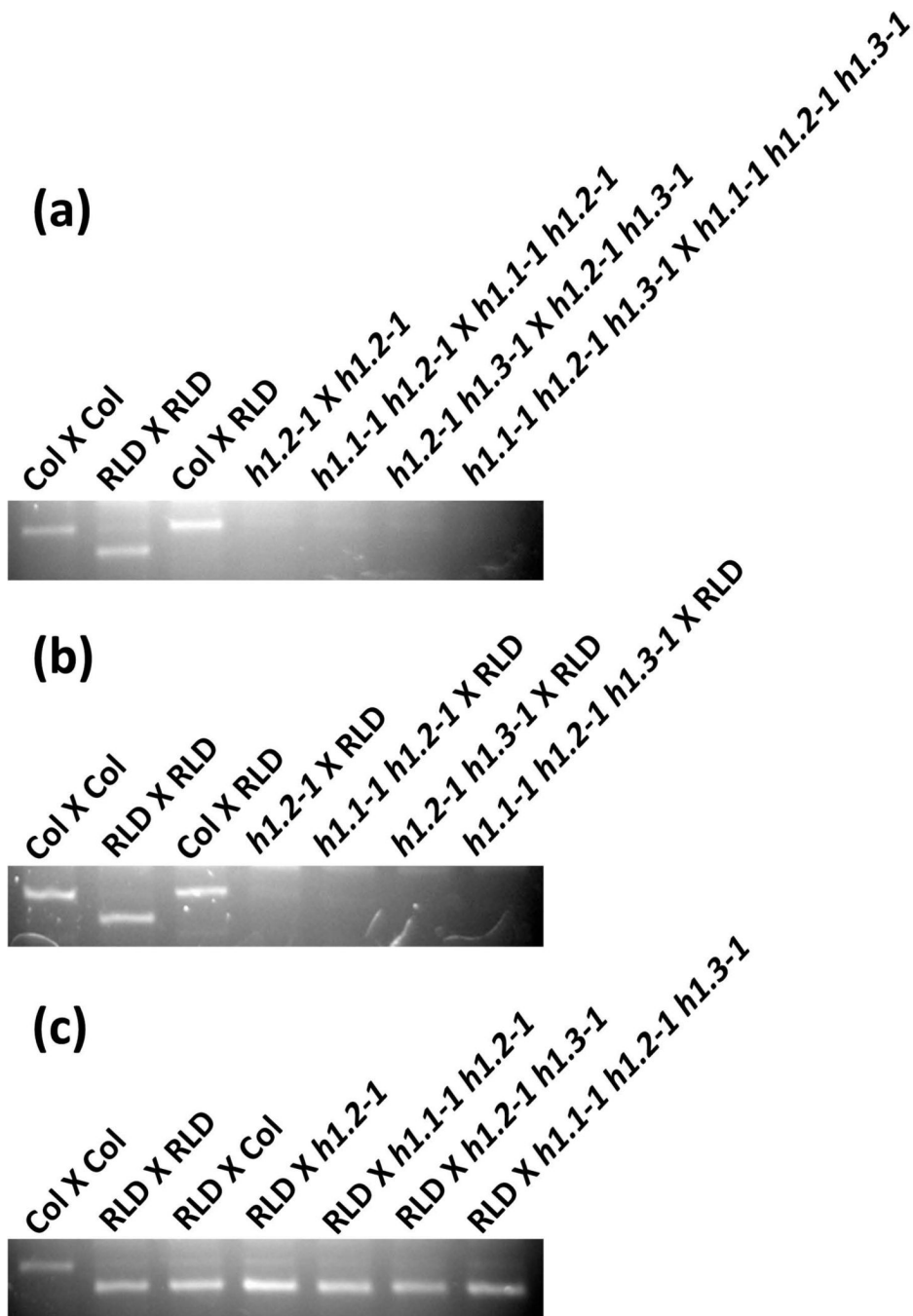


**Figure 2. Three histone *H1* genes *H1.1*, *H1.2*, and *H1.3* in *Arabidopsis***

(a) Gene structures of *H1.1*, *H1.2*, and *H1.3* as well as their respective mutant alleles are shown. Filled and open rectangles represent exons and 5' untranslated region (UTR), respectively. Lines represent introns or promoter. ATG and TGA indicate the translational start and stop codons, respectively. Arrows indicate the locations of T-DNA insertion. *h1.1-1* has a T-DNA insertion in the first exon (+ 215 bp relative to transcription start). *h1.2-1* and *h1.3-1* have a T-DNA insertion in the promoter (– 382 bp and – 62 bp relative to transcription start, respectively).

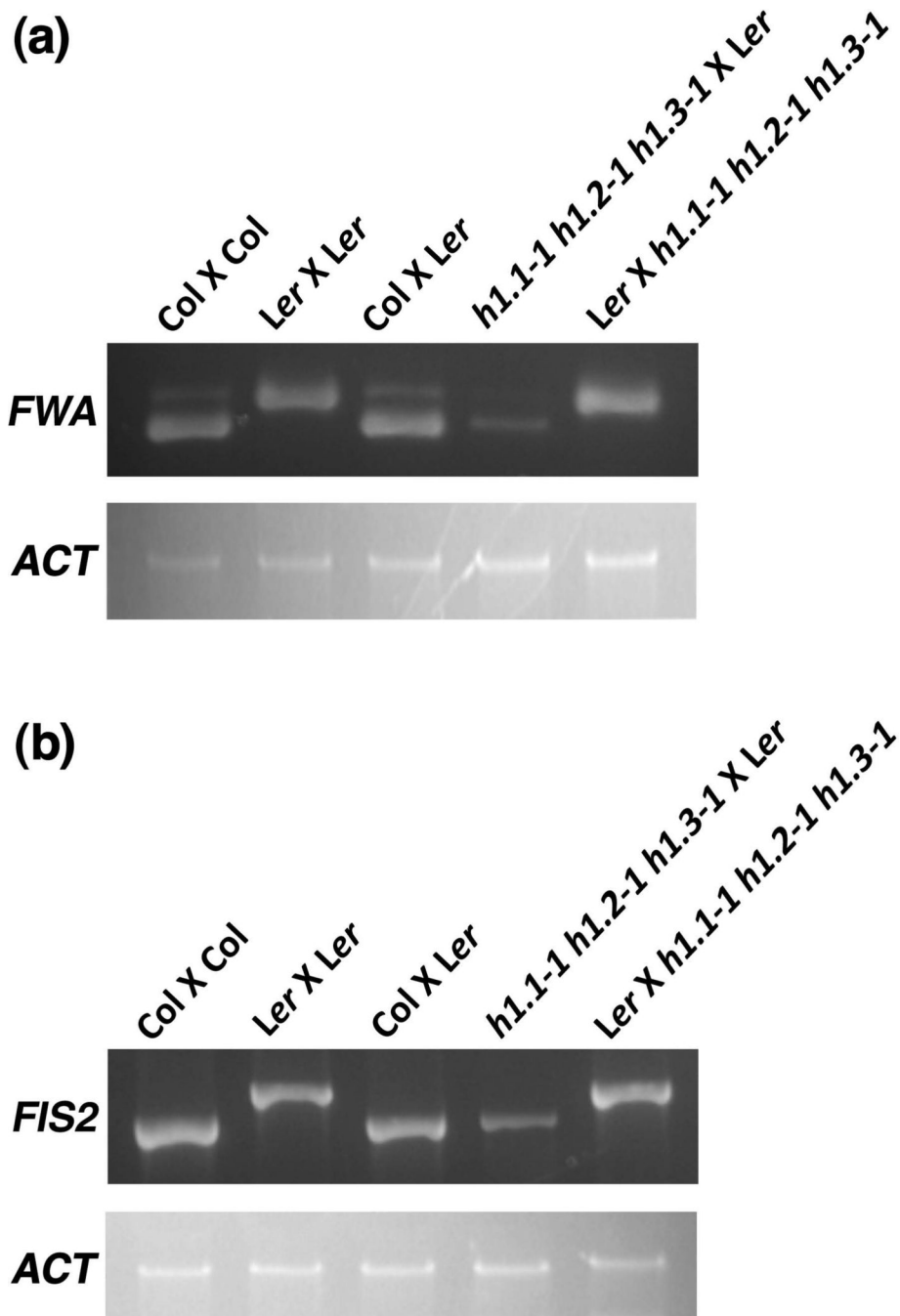
(b) Expression of histone *H1.1*, *H1.2*, and *H1.3* in their respective mutants. Endosperm tissues from seed at 8-DAP of respective genotypes were collected and total RNA was

isolated. Gene expression was examined by using semi-quantitative RT-PCR. *ACTIN* (*ACT*) was used as a control for the experiment.

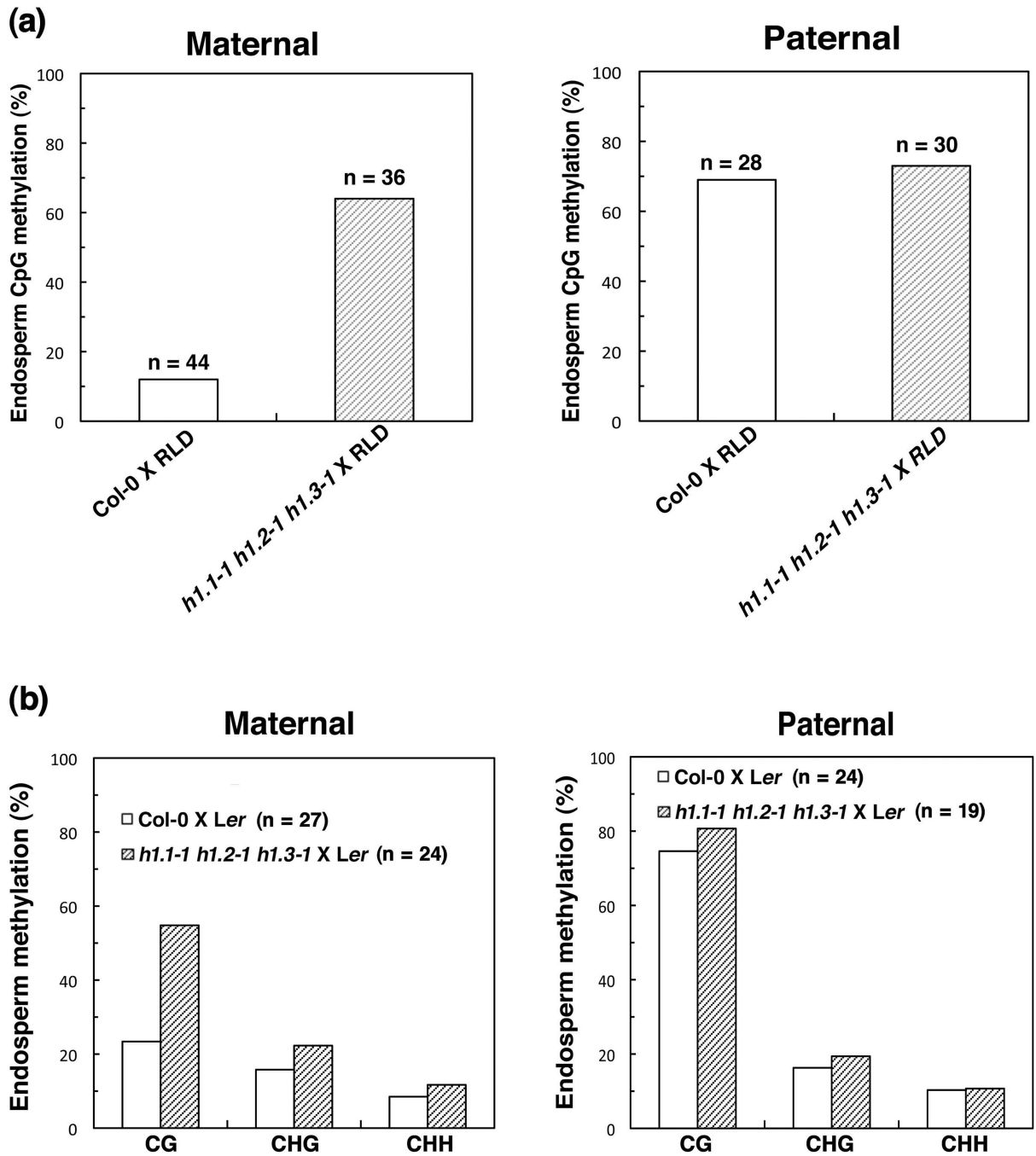


**Figure 3. Mutations in histone *H1* affect *MEA* imprinting in *Arabidopsis* endosperm**  
 (a) *MEA* expression is reduced or undetected in self-pollinated histone *h1* mutants.  
 (b) The maternal histone *h1* mutation diminishes or abolishes *MEA* expression in endosperm.  
 (c) The paternal histone *h1* mutation has no effect on expression of *MEA* in endosperm.  
 Endosperm tissues from seed at 8-DAP of respective crossed siliques were collected and total RNA was isolated. All *h1* mutants are in Col-0 background and the maternal parent was shown first in the crosses (♀ X ♂). The *MEA* alleles from Col-0 and RLD were distinguished by a dCAPS marker. *MEA* expression was examined by using semi-quantitative RT-PCR.





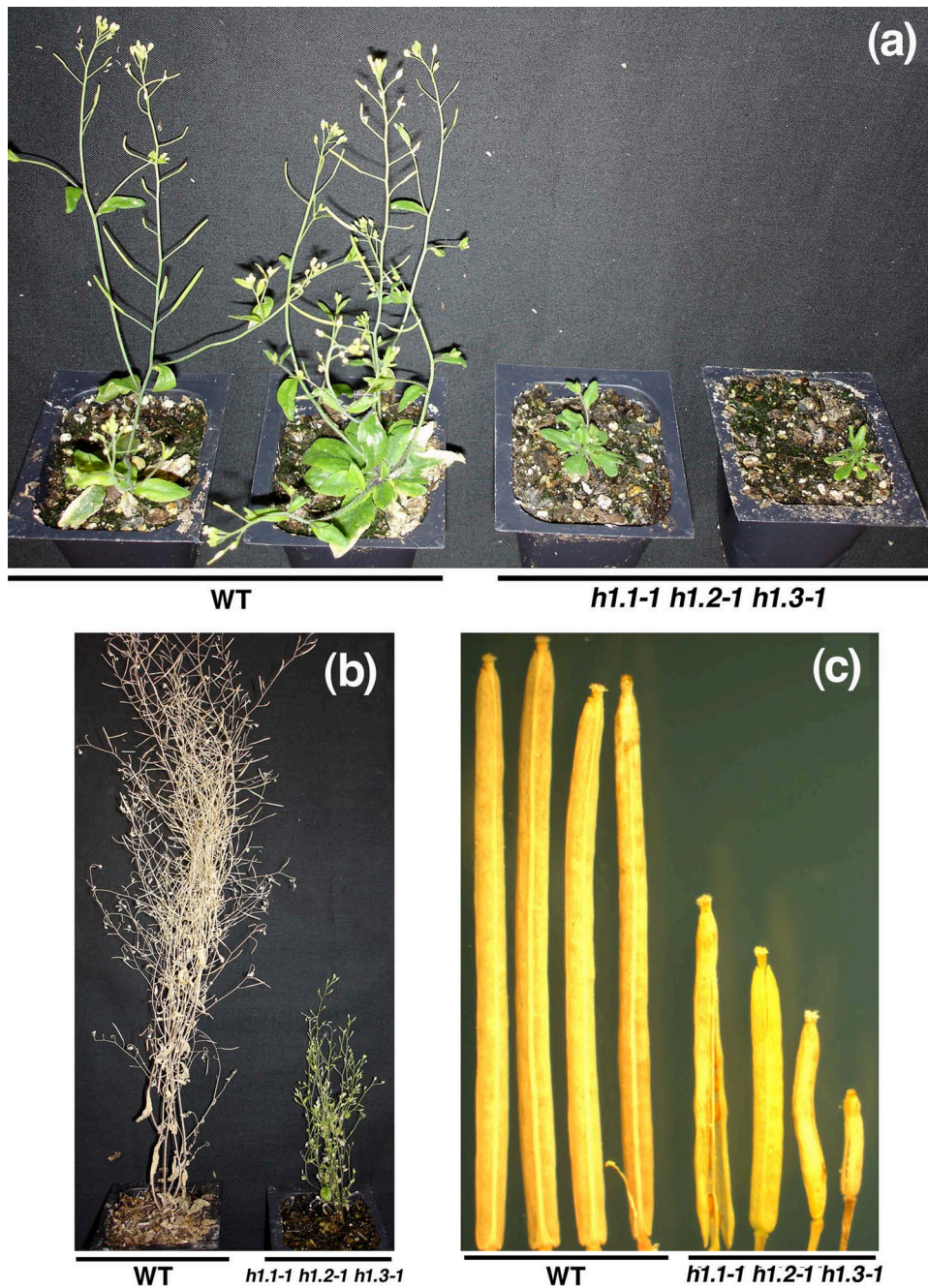
**Figure 4. Mutations in histone *H1* affect *FWA* and *FIS2* imprinting in *Arabidopsis* endosperm**  
 (a) The maternal histone *h1* mutation diminishes maternal *FWA* expression in endosperm.  
 (b) The maternal histone *h1* mutation reduces maternal *FIS2* expression in endosperm.  
 Endosperm tissues from seed at 8-DAP of respective crossed siliques were collected and total RNA was isolated. All *h1* mutants are in Col-0 background and the maternal parent was shown first in the crosses (♀ X ♂). The *FWA* and *FIS2* alleles from Col-0 and *Ler* were distinguished as described (Kinoshita *et al.*, 2004; Jullien *et al.*, 2006a). *FWA* and *FIS2* expression was examined by using semi-quantitative RT-PCR. *ACTIN* (*ACT*) was amplified from the cDNA as a control.



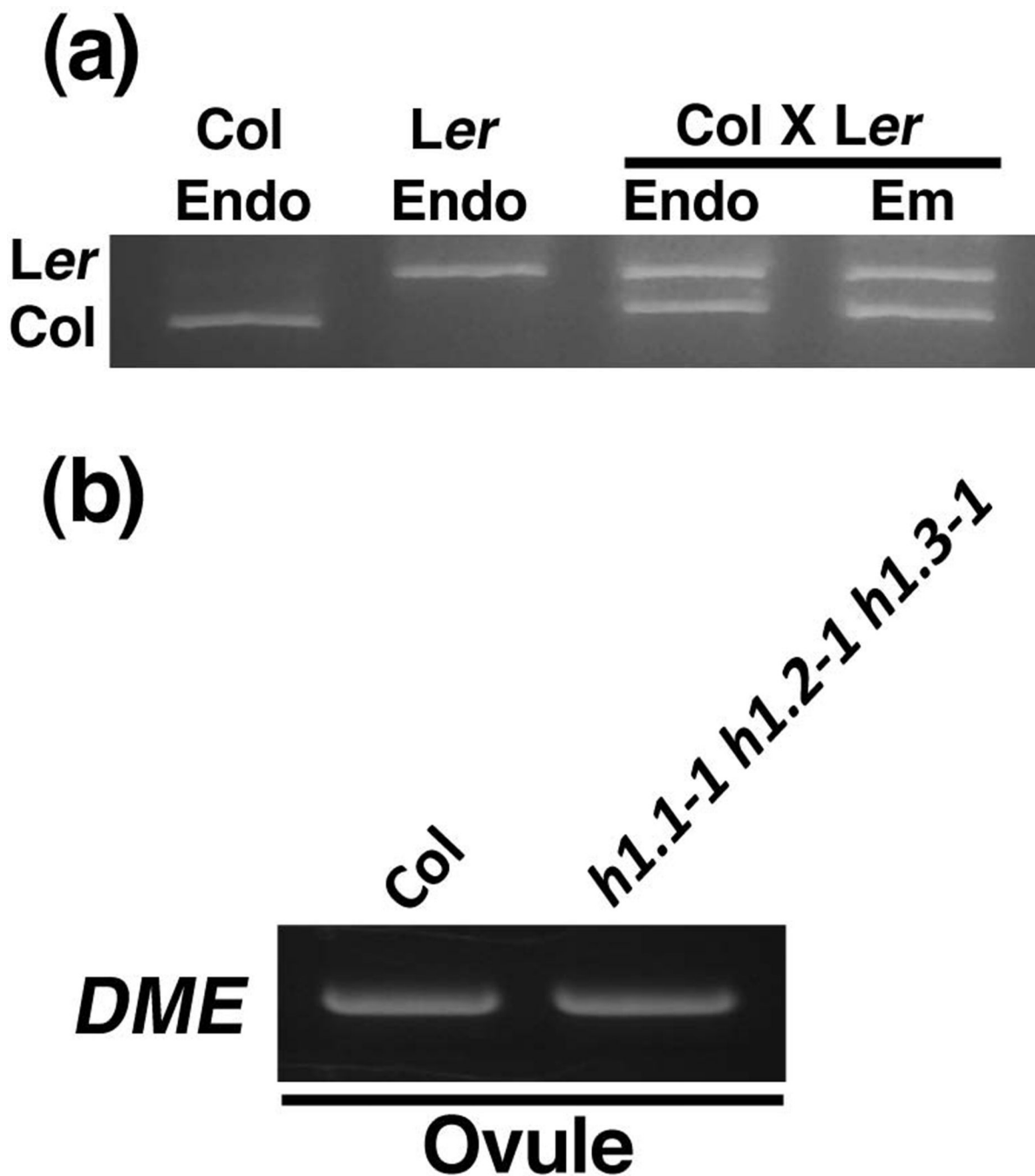
**Figure 5. Histone *H1* mutations result in hypermethylation of the maternal *MEA* and *FWA* allele in endosperm**

(a) The maternal histone *h1* mutation causes hypermethylation of the maternal *MEA* allele in endosperm. DNA methylation profile of the maternal and paternal *MEA* allele in -500 bp in endosperm of crossed seeds was shown. DNA was isolated from endosperm, treated with bisulfite, PCR amplified, cloned, and sequenced. Bisulfite sequencing results are from the genomic endosperm DNA of the crossed siliques of four different triple mutant *h1.1-1 h1.2-1 h1.3-1* plants. “n” represents the number of total clones that were sequenced in the bisulfite sequencing.

(b) The maternal histone *h1* mutation results in increased CG methylation of the maternal 5' repeats of *FWA* in endosperm. DNA methylation profile of the maternal and paternal *FWA* allele in 5' repeats in endosperm of crossed seeds were shown. Bisulfite sequencing was done as described above in Figure 5a. "n" represents the number of total clones that were sequenced in the bisulfite sequencing.



**Figure 6. The triple mutant *h1.1-1 h1.2-1 h1.3-1* shows abnormal developmental phenotypes**  
 (a) Wild type Col-0 and the F<sub>4</sub> generation triple mutant *h1.1-1 h1.2-1 h1.3-1* plants at 6 weeks old. The *h1.1-1 h1.2-1 h1.3-1* mutant plant shows delay in flowering and small stature.  
 (b) Wild type Col-0 and the F<sub>4</sub> generation triple mutant *h1.1-1 h1.2-1 h1.3-1* plants at 10 weeks old. The *h1.1-1 h1.2-1 h1.3-1* mutant plant shows delayed flowering, small stature and is almost infertile.  
 (c) Siliques of wild type Col-0 and the F<sub>4</sub> generation triple mutant *h1.1-1 h1.2-1 h1.3-1* plant. The mutant siliques are shorter and have fewer or no seeds.



**Figure 7. *H1.2* is not imprinted in embryo or endosperm and the *H1* mutation does not affect expression of DME in ovules**

(a) Both maternal and paternal *H1.2* alleles were detected in embryo or endosperm of 8-DAP seed in the cross between Col-0 and RLD. A fragment of *H1.2* cDNA was amplified by PCR, and digested with PstI to distinguish a dCAPS marker between Col-0 and RLD at the *H1.2* locus.

(b) DME expression in wild type Col-0 and the triple mutant *h1.1-1 h1.2-1 h1.3-1*. RNA was isolated from the ovule tissues of wild type and the mutant plants and used for RT-PCR examination of DME expression.



**Table 1**Seed abortion caused by the *HI* mutation

Parental Genotype (Female X Male) <sup>a</sup>	Viable Seed	Aborted Seed	Total Seed	Seed Abortion (%)
<i>h1.1-1 h1.2-1 h1.3-1</i> X <i>h1.1-1 h1.2-1 h1.3-1</i>	370	74	444	16.7
<i>h1.1-1 h1.2-1 h1.3-1</i> X <i>Col-0</i>	529	82	611	13.4
<i>Col-0</i> X <i>h1.1-1 h1.2-1 h1.3-1</i>	535	27	562	4.8

<sup>a</sup>The F<sub>3</sub> progeny of the *h1.1-1 h1.2-1 h1.3-1* mutant plants were used in the genetic crosses.