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Genome demethylation and imprinting in the endosperm

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Imprinting occurs in the endosperm of flowering plants. The endosperm, a product of central cell fertilization, is critical for embryo and seed development. Imprinting in the endosperm is mainly due to the inherited differences in gamete epigenetic composition. Studies have also shown that there are differences in genomic DNA methylation patterns between embryo and endosperm. Examining those differences, along with mutations in the DNA demethylase gene *DEMETER*, gives insight into the number of imprinted genes and how an antagonistic relationship between TE defense and gene regulation could evolutionarily affect imprinting establishment. Finally, studies demonstrate that DEMETER demethylase activity influences endosperm chromatin composition, and could possibly enhance DNA *de novo* methylation activity.

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Introduction

Angiosperm seeds are composed of the seed coat, the mature embryo, and the endosperm. The endosperm, which serves as a conduit to store and transport nutrients to the embryo during initial phases of seed growth, is a major food source for most of the world. With one exception [1•], all known plant gene imprinting occurs in the endosperm. Hence, this review will concentrate on endosperm imprinting.

Gene imprinting is when two alleles are expressed at different levels depending on their parent of origin [2]. This definition includes alleles that are both expressed but at different intensities, and also alleles in which one is expressed and the other is silent. Most of our understanding on the mechanisms of imprinting is from studies of the latter type of imprinting (Table 1) [3,4]. To understand imprinting, it is important to review the different developmental programs of the female and male gametophytes (for a review, see [5,6] and Figure 1). Ultimately, imprinting is often due to the different epigenetic states between the female and male gametes, including differences in DNA methylation.

DNA methylation

Methylation of the fifth carbon of cytosine (5-methylcytosine) is often associated with heterochromatic loci including telomeres and centromeres, and is also coincident with many silent loci, particularly repetitive sequences and transposable elements. In plants, 5-methylcytosine is found in three sequence contexts: CG, CHG, and CHH (H is A, C, or T) [7]. The symmetry of CG and CHG sites allows the template strand to guide methyltransferase to the new unmethylated strand after DNA replication, a process known as maintenance of methylation. METHYLTRANSFERASE 1 (MET1) is required to maintain methylation at CG sites and CHROMOMETHYLASE 3 (CMT3) is required to maintain methylation of CHG sites. Owing to the asymmetry of CHH sites, methylation must always be *de novo* after each round of DNA replication. DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) is required for *de novo* methylation of CHH and, to an extent, CHG sites. The *de novo* methyltransferase is guided to target sites by components of the RNA-directed DNA methylation (RdDM) pathway. For a review of DNA methylation and RdDM, please read Law and Jacobsen [8]. It is important to note that small interfering RNAs (siRNAs), processed from longer double strand RNA (dsRNA), can guide DRM2 to homologous DNA sites for *de novo* methylation. A pool of siRNAs must be maintained to ensure *de novo* methylation after each round of DNA replication.

Demethylation and imprinting

The establishment of imprinting often requires a loss of maternal methylation in the central cell [9,10,11•,12]. Loss of methylation can occur either passively or actively. Passive demethylation occurs if there is loss of methyltransferase activity, resulting in a failure to maintain DNA methylation after DNA replication [13]. It has been shown that during the late stages of female gametophyte development, MET1 expression is down regulated, which could result in a loss of CG methylation in the mature central cell [11•]. Passive demethylation could play an important role in the establishment of imprinting.

Active demethylation requires proteins that can recognize 5-methylcytosine and enzymatically remove it. In plant reproductive organs this is accomplished by DEMETER

Table 1

List of imprinted genes in flowering plants.

	Endosperm allele expressed	Potential function	References
Arabidopsis thaliana			
MEDEA (MEA)	Maternal	Polycomb group complex SET-domain protein	[33,34]
FWA	Maternal	Homeodomain transcription factor	[12]
FIS2	Maternal	Zinc-finger transcription factor	
MPC	Maternal	C-terminal domain of poly(A) binding proteins (PABPs)	[35]
FH5	Maternal	Actin regulator	[36]
MYB3R2	Maternal	Putative c-myb-like transcription factor	[14••]
HDG8	Maternal	Homeobox-leucine zipper transcription factor	[14••]
HDG9	Maternal	Homeobox-leucine zipper transcription factor	[14••]
HDG3	Paternal	Homeobox-leucine zipper transcription factor	[14••]
PHE1	Paternal	MADS-box transcription factor	[26]
At5g62110	Paternal	Putative transcription factor	[14••]
Zea mays			
R-mottled allele[a]	Maternal	Transcription factor	[37]
dzr1[b]	Maternal	Regulates zein protein accumulation	[38]
fie1	Maternal	Polycomb group complex	[39,40,41]
fie2	Maternal	Polycomb group complex	[39,40]
nrp1	Maternal	Unknown	[42]
mez1	Maternal	Polycomb group complex	[43]
meg1	Maternal	Cysteine-rich peptide	[44]
peg1	Paternal	Unknown	[45]

[a] When inherited paternally, kernel has a mottled color pattern. When inherited maternally, kernel is fully colored.
[b] Allele in the MO17 ecotype is imprinted.

(DME), a bifunctional DNA glycosylase/lyase that works in conjunction with the base excision repair pathway to remove 5-methylcytsine and replace it with cytosine [9,10]. Expression of *DME* is detected just before the two haploid polar nuclei fuse, but it quickly disappears after fertilization of the diploid central cell with a haploid sperm cell. DME activity in the central cell is critical for seed development after fertilization because seeds with a maternal *dme* mutant allele abort development and are inviable. *DME* expression is not detected in the pollen, and inheriting a mutant paternal *dme* allele does not affect seed development [9,10]. Because *DME* is expressed in the central cell, and not the pollen, the endosperm inherits genomes with different epigenetic states, which is critical for imprinting establishment.

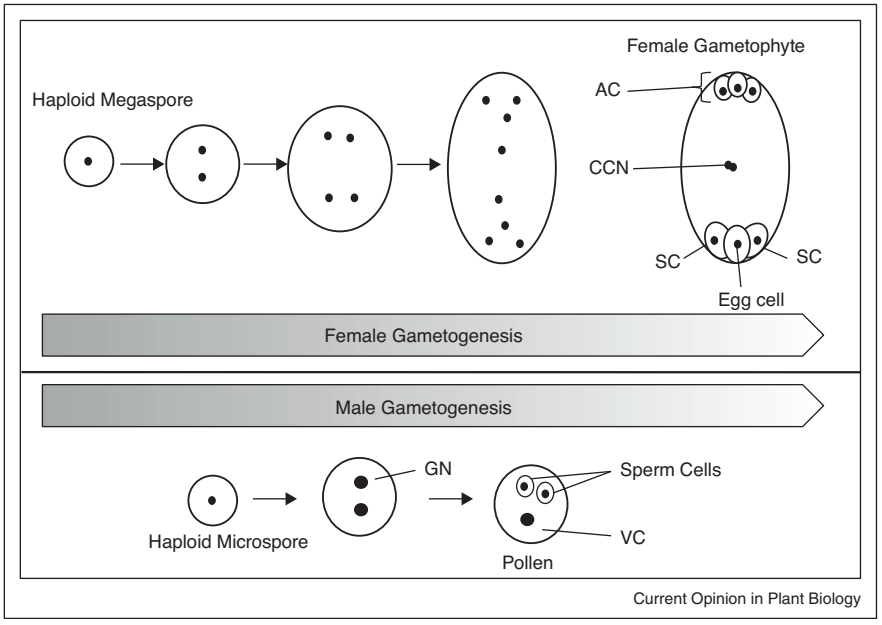
Genomic demethylation and imprinting establishment

To identify DME-mediated imprinted genes, Gehring *et al.* [14••] immunoprecipitated methylated DNA, which was sequenced by high-throughput methods. Comparing embryos, endosperms, and endosperms that inherited a *dme* mutant maternal allele (henceforth will be referred to as '*dme* mutant endosperm'), it was found that the endosperm genome is hypomethylated relative to the embryo, and that methylation increases in *dme* mutant endosperm. In addition, regions with the greatest difference in methylation between endosperm and embryo were regions enriched for transposable elements (TEs) and known siRNA targets. Many of those sites were also located in the proximal regions around genes, including the known imprinted gene *MEDEA* (*MEA*).

Because of the preponderance of differentially methylated regions between embryo and endosperm that also corresponded with TE sites, Gehring *et al.* suggested that imprinting could be a by-product of TE defense [15]. TEs are potential sites for DNA methylation and silencing [16]. If they insert into, or near, a gene promoter, the methylation could inadvertently silence that gene's expression. In the central cell, DME removes methylation from the maternal allele and activates expression. Because DME is not expressed in the pollen, DNA methylation remains resulting in endosperms inheriting two parental genomes with differential DNA methylation (maternal hypomethylated, paternal hypermethylated) and allowing for the establishment of imprinting. With this idea, Gehring *et al.* [14••] identified regions with the greatest methylation difference between embryo and endosperm, and then analyzed those regions at endosperm expressed genes. The authors discovered five new imprinted genes and predicted approximately 50 additional imprinted genes for the *Arabidopsis* endosperm.

Hsieh *et al.* [17••] concurrently performed shotgun bisulfite sequencing of embryo, endosperm, *dme* endosperm, and four-week-old aerial (above-ground) somatic tissue, using a high-throughput DNA sequencing platform. Bisulfite converts cytosine to uracil, but 5-methylcytosin is protected from such changes [18]. This allows resolution of each 5-methylcytosine base in the genome. Importantly, this enables the examination of DNA methylation in the CG, CHG, and CHH sequence contexts. As with Gehring *et al.* [14••], they also discovered a genome-wide loss of methylation in the endosperm compared to

Figure 1



Female and male gametogenesis. (Top Panel) Haploid megaspore, derived from meiosis, proceeds through three rounds of mitosis. After the last mitosis, cell walls form, creating a seven celled structure: the haploid egg cell, two synergid cells (SC), three antipodal cells (AC), and diploid central cell nuclei (CCN). (Bottom Panel) Haploid microspore, derived from meiosis, proceeds through two rounds of mitosis. After the first round, there is a single cell with two haploid nuclei: vegetative nucleus and the generative nucleus (GN). After the second mitotic event, the generative nucleus divides and forms two haploid sperm cells that remain encapsulated in the vegetative cell (VC).

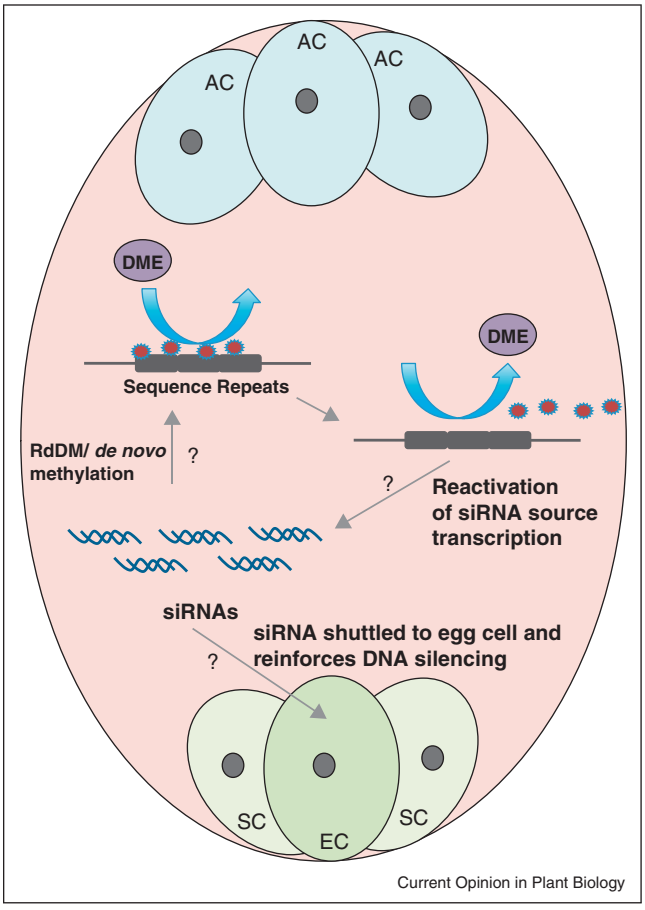
embryo, and that DNA methylation was increased in *dme* mutant endosperm to the level found in aerial tissue [17••]. Because DME is a demethylase, it was anticipated that DNA methylation would increase in *dme* mutant endosperm, which was found at CG sites. Unexpectedly, methylation in *dme* mutant endosperm at CHG and CHH sites was less than wild type endosperm. Many of those sites that were reduced in methylation were also known target sites for siRNAs/RdDM. The authors hypothesized that the removal of DNA methylation by DME, activates expression of siRNAs, which in turn utilize the RdDM pathway for *de novo* non-CG methylation (Figure 2). In the *dme* mutant, there is higher CG methylation, less siRNA activation, which reduces the pool of siRNAs, resulting in a reduction of both the RdDM activity and non-CG methylation. An examination of the siRNA pool in developing seeds supports this hypothesis. A large population of siRNAs in developing seeds is both endosperm specific and imprinted, expressing primarily from the maternal genome [19••]. This suggest that not only could protein-coding genes be imprinted, but also any expressing region, including siRNA loci.

Significance of endosperm maternal genome-wide demethylation

Evolutionarily, why would genome-wide demethylation, which includes the demethylation and possible reactivation of TEs, be beneficial? One possible explanation is that regions require demethylation in the central cell for proper endosperm imprinting and development after fertilization. Because the DNA demethylation is not targeted to imprinted genes, but is genome wide, it could result in widespread TE reactivation. TEs, if reactivated, have the ability to cause unfavorable mutations [20]. However, since the endosperm is a terminal tissue, TEs activated by DME are not inherited by the embryo, and consequently would not be strongly selected against.

An intriguing hypothesis is that genome-wide demethylation activates TEs and other siRNA loci in the endosperm, and that the newly created siRNAs can be transported to the egg or embryo to reinforce TE silencing (Figure 2) [17••]. These ideas imply a positive selection of a siRNA source during evolution, which would benefit the embryo by reinforcing repetitive DNA silencing, and whose genomic danger imposed by a possible TE reactivation would be confined to the endosperm, a terminal tissue. Although the endosperm collects and distributes nutrients to the embryo [21], and siRNAs can have cell-non-autonomous affects in other parts of the plant [22], it remains to be shown that siRNAs could also be transported between the central cell/endosperm and the egg/embryo. A similar siRNA silencing effect was reported in pollen. Slotkin *et al.* [23•] showed that during pollen development (Figure 1), there is a loss of methylation and TE reactivation in the vegetative

Figure 2



Model of genome-wide demethylation. DME removes DNA methylation in the central cell, causing silent genes to begin expression. Many demethylated sites are also repetitive sequences and transposable elements. Model suggests that demethylation of siRNA source loci, including repetitive DNA and transposable elements, would express. The RdDM pathway would process these RNAs to siRNA, and guide *de novo* methylation. siRNAs could also be shuttled to the embryo where the RdDM pathway can reinforce silencing of transposable element and other repetitive DNA sequences. AC, antipodal cells; EC, egg cell; SC, synergid cells.

nucleus (a terminal cell). The authors demonstrated that the TE reactivation allows for additional siRNA production that is shuttled to the two sperm cells to reinforced silencing of siRNA target loci.

Polycomb group complex and imprinting

An additional layer of imprinting regulation involves the polycomb group complex (PcG). The PcG complex was first identified in *Drosophila* and is needed for the suppression of the homeotic or HOX genes [24]. Two distinct complexes exist in *Drosophila*, the PcG repressive complex 1 (PRC1) and the PcG repressive complex 2 (PRC2). In plant sexual reproduction, homologous members of the PRC2 play a significant role [25]. The *Arabidopsis* endosperm PRC2 consists primarily of four proteins: MEA (a SET-domain protein), FERTILIZATION INDEPENDENT ENDOSPERM (FIE, a WD40 protein), FERTILIZATION INDEPENDENT SEED 2 (FIS2, a zinc-finger Su(Z) homolog), and MULTIPLE SUPRESSOR OF IRA1 (MSI1, a p55 homolog). The PRC2 complex methylates lysine 27 on histone H3 (H3K27me), which is associated with compact and silent chromatin. PRC2 is required for the imprinting of *PHERES1* (*PHE1*).

PHE1 is a paternally expressed imprinted gene in the endosperm [26]. PRC2 actively targets the promoter of the maternal *PHE1* allele for H3K27me, which is necessary for maternal allele silencing [27,28]. Mutations that disrupt the PRC2 complex result in *PHE1* biallelic expression. Interestingly, a differentially methylated region located downstream of *PHE1* is thought to be important for imprinting establishment [28,29•]. On the paternal allele, this region requires methylation for endosperm expression and imprinting. This region on the maternal *PHE1* allele is demethylated, and it is thought that this demethylation allows the PRC2 complex to establish maternal allele silencing. Supporting this idea are comparisons between the endosperm H3K27me profile with both the vegetative and endosperm DNA methylation profiles. Weinhofer *et al.* [30], suggest that DNA methylation might prevent the PRC2 complex from binding to target sites, suggesting that DNA demethylation would allow the PRC2 complex to bind and function.

What is the extent of PRC2's involvement in genomic imprinting? Studies of the *cdka;1* mutant suggest that the involvement is quite significant. Pollen carrying a mutation in *cdka;1* will produce a single sperm that can fertilize the egg and not the central cell [31]. After the egg is fertilized, the unfertilized central cell begins to develop into an endosperm-like tissue, which ultimately results in seed abortion. Mutations in *MEA* and *FIE*, both PRC2 genes, will allow the maternal homodiploid endosperm to develop into a viable seed [32]. One explanation is that loss of the paternal contribution disrupts the ratio of maternal to paternal imprinted loci, resulting in seed abortion. If the paternally expressed imprinted alleles that are absent in the unfertilized *cdka;1* endosperm are expressed in the maternal homodiploid genome due to a loss of PRC2-mediated imprinting, seed development is dramatically rescued.

Future directions

To determine the extent imprinting effects seed development, it would be extremely valuable to discover the complete set of imprinted genes, the imprintome. One experimental approach to elucidate the imprintome is to cross different ecotypes, which creates a hybrid endosperm. RNA is isolated, converted to cDNA, and subjected to high-throughput sequencing. In this way, maternal-derived transcripts and paternal-derived transcripts can

be identified by single-nucleotide polymorphisms (SNPs) that distinguish the two ecotypes. RNA transcripts with a significant parental SNP bias represent potential imprinted genes. A similar approach was recently performed in mice, which revealed over 1000 candidate-imprinted genes [46,47]. This approach would complement the above genomic studies that have generated DNA methylation profiles in wild type and *dme* mutant endosperm [14••,17••]. Further genomic studies, including mutations in the PRC2 complex, and possibly the components of the RdDM pathway, will aid in our understanding of the various mechanisms that are needed for imprintome establishment.

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