Regulation of imprinted gene expression in *Arabidopsis* endosperm

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Imprinted genes are expressed primarily or exclusively from either the maternal or paternal allele, a phenomenon that occurs in flowering plants and mammals. Flowering plant imprinted gene expression has been described primarily in endosperm, a terminal nutritive tissue consumed by the embryo during seed development or after germination. Imprinted expression in *Arabidopsis thaliana* endosperm is orchestrated by differences in cytosine DNA methylation between the paternal and maternal genomes as well as by Polycomb group proteins. Currently, only 11 imprinted *A. thaliana* genes are known. Here, we use extensive sequencing of cDNA libraries to identify 9 paternally expressed and 34 maternally expressed imprinted genes in *A. thaliana* endosperm that are regulated by the DNA-demethylating glycosylase DEMETER, the DNA methyltransferase MET1, and/or the core Polycomb group protein FIE. These genes encode transcription factors, proteins involved in hormone signaling, components of the ubiquitin protein degradation pathway, regulators of histone and DNA methylation, and small RNA pathway proteins. We also identify maternally expressed genes that may be regulated by unknown mechanisms or deposited from maternal tissues. We did not detect any imprinted genes in the embryo. Our results show that imprinted gene expression is an extensive mechanistically complex phenomenon that likely affects multiple aspects of seed development.

epigenetics | gene imprinting | angiosperm reproduction | DNA demethylation

Genomic imprinting, the differential expression of alleles of the same gene depending on parent of origin, independently evolved in mammals and flowering plants (1). Imprinted expression is a clear example of inheritance of epigenetic states, because genetically identical sequences are differentially transcribed depending on the sex of the parent from which the gene originates. A widely accepted evolutionary explanation of genomic imprinting is the parental conflict theory (1–3), which argues that, when females mate with multiple males and allocate resources directly to the developing embryo, males will favor expression of genes that maximize resource extraction for their offspring, whereas females will favor genes that equalize resource allocation to all offspring.

Double fertilization is unique to flowering plants and underlies the distinctive cellular programming of plant gene imprinting (4). In the ovule, a haploid megaspore undergoes three mitoses to form the female gametophyte with egg, central, synergid, and antipodal cells. The central cell initially has two haploid nuclei that fuse to create the diploid central cell. In stamens, haploid microspores undergo cell divisions to produce the male gametophyte with two sperm cells and a vegetative cell, which transport the sperm to the egg and central cell. The diploid embryo and triploid endosperm develop from the fertilized egg and central cell, respectively. In flowering plants, all known cases of imprinted expression, with one exception, occur in endosperm (5, 6). Endosperm is a nutrient tissue, acquiring and storing resources from the maternal chalazal seed coat and underlying vasculature to nourish the embryo (7, 8) (Fig. 1), and thus, is the tissue where conflict over resource allocation would be expected to unfold.

Imprinted expression of all known plant genes depends on differential DNA methylation, activity of polycomb repressive complex 2 (PRC2), or both. Maternally inherited mutations in *Arabidopsis thaliana* genes that encode PRC2 proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE; WD40 protein), MULTICOPY SUPRESSOR OF IRA 1 (MSI1; WD40 protein), FERTILIZATION INDEPENDENT SEED 2 (FIS2; zinc finger protein), and MEDEA (MEA; SET domain protein that methylates H3K27) cause endosperm overproliferation, embryo abortion, and seed lethality (9). The *MEA* gene is self-imprinted, with maternal MEA protein activity required to silence the paternal allele after fertilization (10). Maternal PRC2 proteins also silence the paternal allele of the actin regulator, *ARABIDOPSIS FORMIN HOMOLOG 5* (*FH5*) (11).

Active DNA demethylation is catalyzed by the DNA glycosylase DEMETER (DME) that excises 5-methylcytosine in the *A. thaliana* central cell (10). The maternal alleles of *FIS2*, *FLOWERING WAGENINGEN/HOMEODOMAIN GLABROUS 6* (*FWA/HDG6*) homeodomain leucine zipper transcription factor gene, and *MATERNALLY EXPRESSED PAB C-TERMINAL* (*MPC*) polyA binding protein gene are activated by this demethylation. These genes are biallelically expressed in the endosperm if the male (sperm) genome is demethylated by mutation of the *MET1* DNA methyltransferase (12–14). Passive DNA demethylation caused by inhibited expression of *MET1* during female gametophyte cell proliferation might also contribute to imprinted expression (15). Because *FIS2* activation is mediated by DME-dependent DNA demethylation, proper imprinting of genes regulated by PRC2 may also require DME.

Three paternally expressed imprinted transcription factor genes, *HDG3*, *MYB THREE REPEAT 2 (MYB3R2)*, and *PHERES 1* (*PHE1*), have been identified (16, 17). Silencing of the maternal *PHE1* allele depends on a functional PRC2 complex, and maternally inherited mutations in PRC2 cause biallelic expression of *PHE1* (18, 19). In addition, silencing of the maternal *PHE1* allele is thought to require maternal demethylation at the *PHE1* gene (17, 20).

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Data deposition: The sequence reported in this paper has been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE24644).

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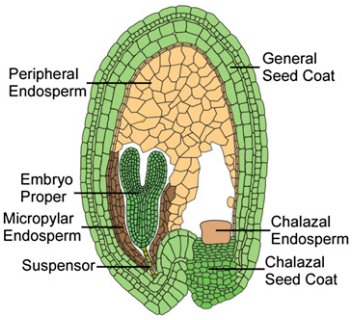

Fig. 1. Drawing of an *A. thaliana* seed with a linear cotyledon stage embryo showing the major seed compartments.

Hundreds of mammalian imprinted genes have been described that are thought to regulate nutrient transfer capacity of fetal placenta, embryonic growth, childhood development, and adult brain function (21, 22). Imprinting disorders affect fetal growth, hormone systems after birth, and behavior. By contrast, only 11 imprinted genes are known in *A. thaliana*, some of which control growth and likely influence nutrient transfer capacity of the endosperm (5). Here, we report identification of imprinted *A. thaliana* genes by deep sequencing of cDNA libraries from polymorphic F1 seeds. We discovered 43 genes regulated by the DNA-demethylating glycosylase DME, the DNA methyltransferase MET1, or the core Polycomb group (PcG) protein FIE that are preferentially expressed from either the paternal or maternal allele in endosperm, including transcription factors, proteins involved in auxin and ethylene signaling, components of the ubiquitin-26S proteosome pathway, regulators of histone and DNA methylation, and small RNA pathway proteins. We also identified maternally expressed genes for which allele-specific expression was not obviously altered by mutations affecting DNA methylation or PcG function, suggesting that paternal silencing of these genes might be caused by an unknown pathway or that the mRNA is deposited in endosperm from maternal tissues. In contrast to endosperm, we did not identify any imprinted genes in embryo. Our study has significantly expanded the known set of imprinted genes in plants, showing that imprinting is a major epigenetic process affecting endosperm gene expression.

Results

Identification of Genes Imprinted in Endosperm. To identify imprinted genes, we prepared cDNA libraries from endosperm derived from two pairs of reciprocal crosses between the Col and L*er* accessions (two independent library pairs). cDNA libraries were sequenced using the Illumina GA2 platform and aligned to both Col and L*er* genomic scaffolds (Dataset S1 and *SI Methods*). Reads that preferentially aligned to either scaffold were assigned to that accession. Each gene received Col and L*er* expression scores equal to the number of reads assigned to each ecotype.

To gauge the performance of our method, we examined all 11 genes previously shown to be imprinted in endosperm (Table S1). Two of these genes (*MEA* and *PHE1*) lack SNPs between Col and L*er*, and another (*MPC*) lacked reads. With the partial exception of *FH5*, other genes behaved consistently with published results, although most lacked sufficient reads for statistical significance (Dataset S2). For example, *HDG3* is paternally expressed in both crosses, *HDG9* and *MYB3R2* are maternally expressed in both crosses, and *HDG8* is maternally expressed when Col is female (CxL) and biallelic when L*er* is female (LxC) (Table S1), as previously described (16). *FH5* was reported as reciprocally maternally expressed in a cross between the L*er* and C24 ecotypes (11), whereas we find that it is maternally expressed when Col is female but biallelic when L*er* is female, a discrepancy that may be explained by the different ecotypes used.

At a *P* value cutoff of 0.05, we identified 1,081 genes preferentially or exclusively expressed from the female genome and 25 genes preferentially or exclusively expressed from the male genome in endosperm (Dataset S2), with 739 maternally expressed and nine paternally expressed genes at a stricter *P* value cutoff of 0.001 (Fisher's exact test) (Dataset S2, and Fig. S1). A potential complication in identifying genes expressed from the female genome is that contamination with RNA from maternal tissues such as the seed coat will mimic imprinting. Indeed, several genes in our $P < 0.001$ maternal dataset, such as *TRANSPARENT TESTA 10* (*At5g48100*), are highly expressed in the seed coat (Dataset S2). To address this issue, we sequenced cDNA derived from CxL F1 endosperm tissue obtained through laser capture microdissection (LCM), a technique that allows much greater precision than manual dissection. We considered genes to be imprinted if their expression levels in both of our manually dissected endosperm library pairs were no more than fourfold greater than those from the LCM dataset. We also included genes close to the above cutoff if their imprinted status was significantly altered by mutations that would not affect expression in maternal tissues (*met1*, *fie*, and *dme*). These filtering steps reduced the number of maternally expressed imprinted genes to 114 ($P < 0.001$) (Dataset S2), which includes two previously reported genes, *MYB3R2* and *HDG9* (Table S1). We focused further analyses on the LCM-filtered maternal $P < 0.001$ set and the paternal $P < 0.001$ set,

Table 1. Selected maternal genes

Number	Annotation	CxL M/P	LxC M/P	*met1* M/P	*fie* M/P	*dme* M/P	Endo exp*	Emb exp*	*met1* exp	*fie* exp	*dme* exp
AT1G59930 m/d	PHE-related	495/1	465/1	202/189	1,418/5	32/1	418/1,123	22/0	1,854	3,287	93
AT2G17690 m/d	SDC	124/1	119/2	33/87	111/3	10/0	59/96	4/2	186	66	8
AT2G24740 #	SUVH8	48/0	50/19	3/1	248/1	77/0	31/24	1/0	19	178	40
AT2G28380 m/d/f	DRB2	806/198	737/171	94/60	37/5	69/11	936/343	789/490	562	156	272
AT2G34880 #/d	JMJ15	3/0	12/0	0/0	14/0	0/0	8/3	1/2	3	24	2
AT4G00220 m/d/f	JLO	666/11	836/15	116/45	22/1	36/1	478/186	33/1	469	33	37
AT4G18650 m/d/f	TF-related	807/89	92/14	25/72	2/1	1/0	434/32	73/3	416	6	4
AT4G31060 m/d/f	ERF/AP2 TF	719/9	229/7	45/62	0/0	17/0	498/150	41/25	472	2	13
AT5G03280 m/d/f	EIN2	1,346/207	1,725/169	155/80	305/67	269/65	730/610	212/84	393	200	142
AT5G35490 m/d/f	MRU1	43/0	30/0	4/6	3/2	0/0	32/16	0/0	23	3	1

A partial list of previously undescribed maternally expressed imprinted genes at a $P < 0.001$ (except #). Total maternal (M) and paternal (P) reads are shown for the indicated genotypes as well as transcriptional scores (number of reads per kilobase of sequence per 10 million aligned reads) for endosperm (Endo exp), embryo (Emb exp), and the indicated mutant genotypes. #, identified by Sanger sequencing; m, biallelic in *met1* endosperm; d, down-regulated in *dme* endosperm; f, down-regulated in *fie* endosperm; TF, transcription factor.
*Transcriptional scores derived from manually dissected and LCM tissue are shown before and after the slash, respectively.

Table 2. Paternally expressed imprinted genes

Number	Annotation	CxL M/P	LxC M/P	*met1* M/P	*fie* M/P	*dme* M/P	Endo exp*	Emb exp*	*met1* exp	*fie* exp	*dme* exp
AT1G17770 #/F/D/M	SUVH7	3/5	5/13	0/0	368/213	295/254	3/6	0/0	0	159	121
AT1G31640 F/M	AGL92	1/6	1/14	0/0	150/131	8/49	2/16	1/0	0	115	14
AT1G48910 F/D/M	YUC10	36/70	30/279	6/5	124/131	703/372	78/2	0/0	15	194	651
AT1G57800 F/D	VIM5	249/2,818	343/3,513	10/246	3,734/3,966	1,066/694	458/208	22/2	157	1,965	324
AT1G60410 F/M	F-box	4/33	6/32	0/0	552/301	17/160	8/10	3/0	0	351	56
AT2G21930 F/M	F-box	2/15	0/18	0/0	16/24	0/7	17/4	1/3	0	109	17
AT2G36560 F/D/M		0/55	3/42	0/0	826/227	84/334	8/17	1/0	0	263	78
AT4G11940 F/D		1/6	1/15	0/0	124/141	67/36	3/9	0/0	1	183	54
AT5G63740 F/D		16/34	16/59	0/0	11,420/12,497	1,110/1,391	24/9	7/0	0	15,141	1,159

A list of previously undescribed paternally expressed imprinted genes at a $P < 0.001$ (except #). Total maternal (M) and paternal (P) reads are shown for the indicated genotypes as well as transcriptional scores (number of reads per kilobase of sequence per 10 million aligned reads) for endosperm (Endo exp), embryo (Emb exp), and the indicated mutant genotypes. #, identified by Sanger sequencing; F, biallelic in *fie* endosperm; D, biallelic in *dme* endosperm; M, paternal allele down-regulated in *met1* endosperm.

*Transcriptional scores derived from manually dissected and LCM tissue are shown before and after the slash, respectively.

because we believe that these best represent genes with imprinted endosperm expression (Tables 1 and 2, Table S1 and S2, and Fig. S1).

We examined allele-specific expression of 52 genes by RT-PCR followed by conventional DNA sequencing, and the results agreed closely with those obtained by sequencing cDNA libraries using the Illumina GA2 platform (Figs. 2*A* and 3*A*, Fig. S2*A*, and Datasets S2 and S3). In both datasets, 43 genes showed clear monoallelic expression in reciprocal crosses, and 9 genes showed clear monoallelic expression in one cross, with a greater tendency to biallelic expression in the reciprocal cross. Similar effects of ecotypes on parent of origin expression have been reported for imprinted genes in *A. thaliana* and mammals (22, 23). We also identified three imprinted genes with a *P* value that was outside our statistical selection criteria, maternally expressed *SUVH8* (*At2g24740*) and *JUMONJI C DOMAIN 15* (*JMJ15*/*PKDM7C*; *At2g34880*) (Table 1 and Fig. S2) and paternally expressed *SUVH7* (*At1g17770*) (Fig. 3 and Table 2). In total, we identified 116 maternally expressed genes (Table 1 and Dataset S2), including 2 that were previously described (*HDG9* and *MYB3R2*) (Table S1), and 10 paternally expressed genes, including previously described *HDG3* (Table 2, Table S1, and Fig. S1).

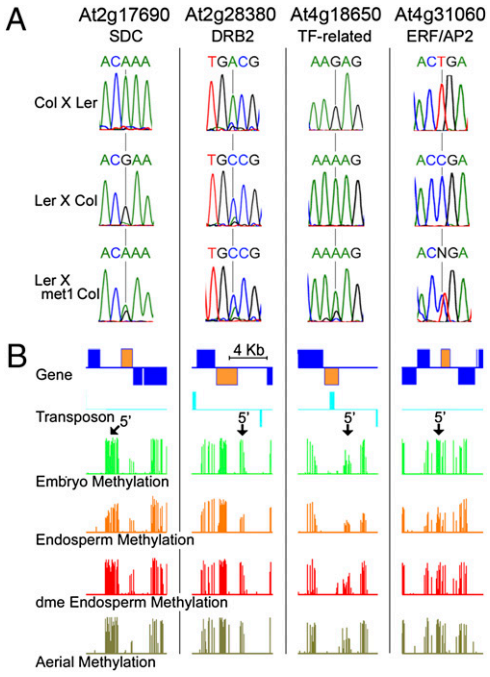

Fig. 2. Maternally expressed imprinted genes. (*A*) RT-PCR sequencing chromatographs at selected SNP regions measuring allele-specific expression in reciprocal crosses between L*er* and Col ecotypes and in female L*er* crossed to male *met1-6* Col-*gl*. (*B*) CG methylation profiles in WT embryo, endosperm, aerial tissues, and *dme* endosperm for genes shown in *A* are displayed. Genes and transposable elements oriented 5′ to 3′ and 3′ to 5′ are shown above and below the line, respectively. Gene models indicated in yellow represent the imprinted genes as shown in *A*. Arrows indicate the 5′ end of imprinted genes where CG demethylation is seen in WT endosperm.

Imprinted Expression Not Detected in Embryo. We prepared embryo cDNA libraries from one pair of reciprocal crosses between the Col and L*er* accessions, sequenced using the Illumina GA2 platform, and processed the sequencing data as we did for endosperm libraries. We identified one paternally expressed gene, *VARIATION IN METHYLATION 5* (*VIM5*) (24), and 37 maternally expressed genes at a *P* value cutoff of 0.001 (Fisher's exact test) (Dataset S2). Erroneous identification of imprinted genes in embryo can be caused by contamination with endosperm or seed coat tissue, a concern highlighted by the fact that *VIM5* is by far the most highly paternally expressed imprinted gene in endosperm (Table 2), and 30 of the maternally expressed genes were identified as imprinted in endosperm at a *P* value below 0.001 before LCM filtering (Dataset S2). To address this issue, we sequenced cDNA derived from CxL F1 LCM-isolated embryos and filtered our embryo dataset as we did for endosperm data. Only two of the putatively imprinted embryo genes (*At1g70830* and *At5g47150*) survived this treatment, and both were discarded, because they were scored as imprinted in endosperm but filtered out as likely maternal tissue contaminants (Dataset S2). Our results indicate that imprinted gene expression is either very rare or does not occur in *A. thaliana* embryos, which is consistent with imprinted genes not being detected in vegetative rice tissues (25) but in contrast with extensive imprinted expression of genes in the mammalian fetus and adult (21, 22). Alternatively, imprinted genes in the embryo may be difficult to detect because of low RNA levels or expression that is restricted to specific cell types or tissues.

Effects of Paternal *met1* and Maternal *dme* and *fie* Mutations on Maternally Expressed Genes. To better understand the mechanisms underlying imprinted gene expression, we sequenced cDNA libraries from endosperm generated from crosses between L*er* and Columbia *glabrous* (Col-*gl*) where the male was homozygous for the *met1-6* mutation, L*er* and Col where the female was heterozygous for *fie-1*, and Col-*gl* and L*er* where the female was heterozygous for *dme-2*. Endosperm with a maternal mutant *dme* or *fie* allele was identified by its abnormal development.

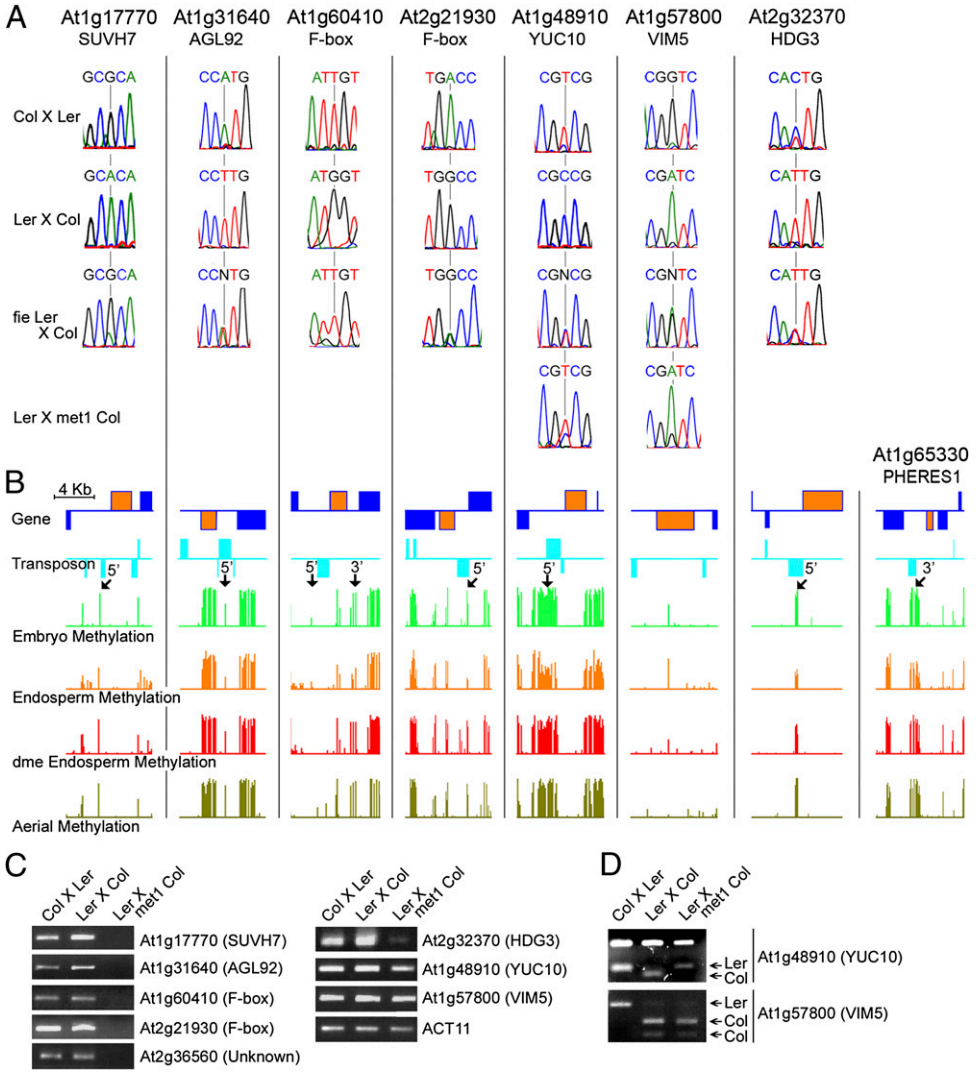

Fig. 3. Paternally expressed imprinted genes. (*A*) RT-PCR sequencing chromatographs at selected SNP regions measuring allele-specific expression in reciprocal crosses between L*er* and Col ecotypes, in female *fie* L*er* crossed to male Col for all genes, and in female L*er* crossed to male *met1-6* Col-*gl*. (*B*) CG methylation profiles of genes shown in *A* and *PHE1* are displayed. Genes and transposable elements oriented 5′ to 3′ and 3′ to 5′ are shown above and below the line, respectively. Gene models indicated in yellow color represent the imprinted genes shown in *A*. Arrows indicate 5′ and 3′ ends of imprinted genes where CG demethylation is detected in WT endosperm. (*C*) Expression analysis by semiquantitative RT-PCR in WT reciprocal crosses between L*er* and Col ecotypes and in female WT L*er* crossed to male *met1-6* Col-*gl*. (*D*) Allele-specific expression of *At1g48910* (*YUC10*) and *At1g57800* (*VIM5*). RT-PCR analysis using F1 endosperm RNA isolated from Col females crossed to L*er* males, L*er* females crossed to Col males, and L*er* females crossed to Col-*gl met1-6* males. For *YUC10* RT-PCR products, HpaII enzyme cuts the L*er* allele into a 212- and 77-bp band, whereas the Col allele is cut into 212-, 53-, and 24-bp bands. For *VIM5* RT-PCR products, enzyme BsmI cuts the Col allele but not the L*er* allele.

RNA was isolated, and libraries were constructed, sequenced, and analyzed as described for WT endosperm above.

Previous studies showed that DNA methylation silences the paternal alleles of the maternally expressed imprinted genes *FWA*, *FIS2*, and *MPC*, and these genes exhibit activation of the paternal allele in endosperm fertilized with *met1* pollen (12–14). Consistent with this model, we identified nine maternally expressed genes that displayed biallelic expression caused by a paternally inherited *met1* mutation (Fig. 2*A*, Table 1, Figs. S1 and S2*A*, and Table S1). Genes affected by *met1* include transcription factors *MYB3R2* and *ERF/AP2* (*At4g31060*), *At1g59930*, which encodes a truncated PHE1-related MADS box transcription factor gene, and three genes known to be regulated by DNA methylation: *SDC* (*At2g17690*) and *MRU1* (*At5g35490*), which are overexpressed in lines lacking non-CG methylation (26, 27), and *At4g18650*, a transcription factor gene down-regulated by mutation of the *DME* homolog *REPRESSOR OF TRANSCRIPTIONAL GENE SILENCING 1* (*ROS1*) in seedlings (28). *SDC* encodes an F-box gene that is predicted to confer specificity to the E3 ligase complex that ubiquitylates proteins targeted for degradation by the 26S proteasome (29). Among *met1*-affected genes are two regulators of hormone signaling: *JAGGED LATERAL ORGANS* (*JLO*; *AT4G00220*), a transcription factor that affects transport of the plant hormone auxin by regulating the expression of *PINFORMED* auxin-efflux carrier genes (30), and *ETHYLENE INSENSITIVE 2* (*EIN2*; *At5g03280*), a membrane protein crucial for perception of the gaseous hormone ethylene that is also required for proper auxin, abscisic acid, jasmonic acid, salicylic acid, and cytokinin signaling (31) (Table 1). *DOUBLE-STRANDED RNA BINDING 2* (*DRB2*; *At2g28380*) is a predicted component of the small RNA pathway (32). Available microarray data (http://seedgenenetwork.net; GEO accession no. GSE12404) show that the *met1*-affected genes are expressed primarily in endosperm (Fig. S3*A*). Although small in

number, many maternally expressed imprinted genes affected by *met1* are likely endosperm-specific key regulators that activate or repress other genes.

Active DNA demethylation catalyzed by DME in the central cell (10) has been implicated in the activation of maternal *FWA*, *FIS2*, and *MPC* alleles, because their maternal allele expression is absent or reduced when a *dme* mutation is maternally inherited (12–14). All nine above-described genes that are biallelically expressed in *met1* exhibited down-regulation of the maternal allele in *dme* endosperm (Table 1 and Table S1). Examination of our previously published DNA methylation data (33) revealed that these genes show DNA methylation that either overlaps or is just upstream of their transcriptional start sites (TSS) and that is reduced in endosperm in a DME-dependent manner (Fig. 2*B* and Fig. S2*B*), which is consistent with the model that DME-mediated DNA demethylation activates maternal allele expression of these genes.

JMJ15 (*At2g34880*) is closely related to *JMJ14* (*PKDM7B*), which is thought to demethylate trimethylated lysine 4 of histone H3 (H3K4me3) and is involved in DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)-mediated maintenance of DNA methylation (34). Maternal *JMJ15* expression was not detected in a *dme* mutant, suggesting that DME is required to demethylate and activate maternal allele expression (Fig. S2*C*). However, the expected activation of paternal allele expression in a *met1* mutant was not detected (Fig. S2*A*). It is possible that other MET1 homologs expressed in the endosperm silence the *JMJ15* paternal allele by maintaining CG methylation in the region of its transcriptional start site (Fig. S2*B*).

Another important regulator that maintains the silent state of paternal alleles of imprinted genes is the maternal PRC2 complex. Previous analysis of imprinted genes that do not exhibit activation of the paternal allele in endosperm fertilized with *met1* pollen (*FH5* and *MEA*) revealed that the maternally expressed PRC2 complex silences the paternal allele (10, 11). In accordance with this idea, we identified 20 genes that exhibited activation of the paternal allele caused by a maternally inherited *fie* mutation (Fig. S1 and Table S2). Other than the SKP2B F-box protein (At1g77000) and two zinc-finger proteins (At1g08050 and At5g22920), most of these genes function in intermediary metabolism or signaling. Metabolism genes encode the ADS2 lipid desaturase (At2g31360), an acylphosphatase (At5g03370) that might function in glycolysis, the TPK5 potassium channel protein (At4g01840), and the FPS1 farnesyl diphosphate synthase (At5g47770) that is in the isoprenoid biosynthesis pathway. Signaling genes encode the PP2C-related protein phosphatase (At3g17250), which may negatively regulate protein kinase pathways, a phosphoinositide binding protein (At3g22810) potentially involved in lipid signaling, and the ACX1 acyl-CoA oxidase (At4g16760) that is in the jasmonate hormone biosynthesis pathway (35). Most of these genes are expressed primarily in the endosperm (Fig. S3*A*).

Two of twenty imprinted genes affected by *fie*, *At1g69900* and *At5g47770* (*FPS1*), display biallelic expression caused by a maternally inherited *dme* mutation (Fig. S1 and Table S2), consistent with the role of DME in activating maternal expression of the core PRC2 components FIS2 and MEA. One possible explanation for a more limited effect of *dme* compared with *fie* might be that *FIE* is a single copy gene required for all PRC2 complex formation (9), whereas *MEA* and *FIS2* are members of gene families. PcG proteins related to MEA (SWINGER and CURLY LEAF) and FIS2 (VERNALIZATION 2 and EMBRYONIC FLOWER 2) are expressed in endosperm (http://seedgenenetwork.net). These proteins can interchangeably form PRC2 complexes (36) and might provide redundant PRC2 functionality in a *dme* mutant background.

We found that the PRC2 complex is required for proper expression levels but not imprinting of some genes (Table 1, Table S1, and Dataset S2). A good example of this is the maternal *FWA* allele, which is activated by DME-dependent DNA demethylation (12). In *fie* mutant endosperm, the maternal allele of *FWA* is massively overexpressed, whereas paternal expression is unaffected (Table S1). These results reveal a hierarchical control of epigenetic marks. For genes like *FWA*, DNA demethylation is required for activation, whereas PRC2 regulates the expression level of the activated allele.

Effects of Paternal *met1* and Maternal *dme* and *fie* Mutations on Paternally Expressed Genes. We identified nine paternally expressed genes (Fig. S1 and Table 2) that are expressed primarily in the endosperm within the seed (Fig. S3*B*). Many of these genes encode potential regulatory proteins (Table 2), including the transcription factor *AGAMOUS LIKE 92* (*AGL92*), *YUCCA10* (*YUC10*), a homolog of monooxygenase enzymes that synthesize auxin (37), and two F-box genes (*At1g60410* and *At2g21930*). SUVH7 (At1g17770) is a SET domain protein related to SUVH4 (KRYPTONITE), SUVH5, and SUVH6 histone H3 lysine 9 (H3K9) methyltransferases required for CHROMOMETHYLASE 3 (CMT3)-mediated non-CG DNA methylation (38), and it is the closest homolog of maternally expressed SUVH8 (39). VARIATION IN METHYLATION 5 (VIM5) belongs to a protein family required for maintenance of CG methylation.

PHE1 is a paternally expressed imprinted gene that is biallelically expressed in endosperm with maternally inherited mutations in PRC2 (18, 19). Maternal demethylation of tandem repeats downstream of *PHE1* is also thought to be required for maternal *PHE1* silencing. This idea is supported by the observation that loss of methylation in the paternal genome because of a *met1* mutation reduced expression of the paternal *PHE1* allele (17, 20). Indeed, we detected DME-dependent endosperm hypomethylation of these tandem repeats (33) (Fig. 3*B*). Thus, the current model explaining regulation of *PHE1* imprinting proposes that maternal DNA demethylation near the gene exposes a PRC2 binding site, thereby allowing PcG-mediated silencing of the maternal allele (17). Supporting this model, Weinhofer et al. (40) recently reported that DNA hypomethylation allows targeting by PcG proteins in endosperm. This model predicts that demethylation of the paternal genome by *met1* should silence similarly regulated genes by exposing the paternal allele to PRC2-mediated repression, whereas a maternal *fie* mutation should cause biallelic expression by disabling PRC2.

The silenced maternal alleles of all nine paternally expressed imprinted genes that we identified and *HDG3* are activated by a maternal *fie* mutation, in some cases (e.g., *At5g63740*) accompanied by massive overexpression of both alleles, indicating that maternal alleles of these genes are silenced by the PRC2 complex in WT endosperm (Table 2, Fig. S1, and Table S1). Paternal allele expression of seven genes (*SUVH7*, *AGL92*, *At1g60410* F-box, *At2g21930* F-box, *YUC10*, *At2g36560*, and *HDG3*) is reduced in endosperm fertilized with *met1* pollen, indicating that DNA methylation is required for WT paternal allele expression and likely prevents the establishment of repressive PRC2 complexes on the paternal allele (Fig. 3*C*). For *YUC10*, the *met1* mutation activates expression of the maternal allele (Fig. 3 *A* and *D*), which was also reported for *PHE1* (20). These results are consistent with the model proposed for *PHE1* regulation. However, the imprinted status of *VIM5* is unaffected by *met1* (Fig. 3 *A* and *D* and Table 2), and little DNA methylation is present at or near the *VIM5* gene (Fig. 3*B*), suggesting that *VIM5* maternal allele repression may be mediated by PRC2 independent of DNA demethylation.

As described above, a *dme* mutation can theoretically lead to activation of the maternal allele, because retention of DNA methylation prevents binding of the repressive PRC2 complex or PRC2 activity is compromised. Among the seven genes that show reduced paternal allele expression in a *met1* mutant, we find that

Table 3. *VIM* and *MET* genes

Number	Annotation	CxL M/P	LxC M/P	*met1* M/P	*fie* M/P	*dme* M/P	Endo exp*	Emb exp*	*met1* exp	*fie* exp	*dme* exp
AT1G57820	VIM1	233/144	168/442	23/51	215/178	289/72	114/47	717/632	55	136	95
AT1G66050	VIM2	22/7	28/24	1/0	206/126	81/85	69/73	74/67	11	856	436
AT5G39550	VIM3	2/17	30/13	1/0	151/20	14/58	35/38	138/141	19	329	111
AT1G66040	VIM4	NA	NA	NA	NA	NA	50/62	56/53	8	678	380
AT1G57800	VIM5	249/2,818	343/3,513	10/246	3,734/3,966	1,066/694	458/208	22/2	157	1,965	324
AT5G49160	MET1	1/0	2/1	2/0	7/0	6/6	87/87	327/375	34	264	287
AT4G08990	MET2	93/13	60/55	1/1	141/81	326/81	36/78	6/4	3	136	130
AT4G13610	MET3	12/17	59/11	2/0	1,204/461	115/479	13/19	1/1	1	434	121
AT4G14140	MET4	176/72	199/117	11/14	619/344	681/222	66/205	7/5	29	261	172

A list of all *A. thaliana VIM* and *MET* genes. Total maternal (M) and paternal (P) reads are shown for the indicated genotypes, as well as transcriptional scores (number of reads per kb of sequence per 10 million aligned reads) for endosperm (Endo exp), embryo (Emb exp), and the indicated mutant genotypes. *Transcriptional scores derived from manually-dissected and LCM tissue are shown before and after the slash (/), respectively.

the *dme* mutation causes biallelic expression of four genes (*SUVH7*, *YUC10*, *At2g36560*, and *HDG3*) (Table 2, Table S1, and Fig. S1). For two genes (*AGL92* and *At1g60410* F-box), maternal allele expression is activated; however, paternal allele expression still predominates (Table 2), which may reflect the complex interactions between DNA methylation and PRC2 function that were reported for the regulation of *PHE1* (20).

Maintenance of CG Methylation in Endosperm Is Carried Out by Distinct *MET* and *VIM* Genes. The *A. thaliana VIM* family is comprised of five genes, of which only *VIM1*, *VIM2*, and *VIM3* are expressed in leaves and flowers (24). These three genes are mostly redundant, and the corresponding triple mutant plants lack CG DNA methylation—the same phenotype as animal cells without the VIM ortholog Uhrf1 (24, 41). Uhrf1 contains an SRA (SET and RING-associated) domain that specifically binds to DNA hemimethylated at CG sites and thus, is postulated to provide the specificity that allows the MET1/Dnnmt1 methyltransferases to maintain CG DNA methylation after DNA replication (42). Although *VIM5* expression is low in leaves, flowers (24), and embryos, *VIM5* is the predominant *VIM* gene in endosperm (Table 3). Thus, the DNA methyltransferases of the MET1 family primarily depend on *VIM5* expressed from the paternal allele to maintain CG DNA methylation after DNA replication in endosperm.

In addition to *VIM5* imprinting, the expression of *MET1* (*At5g49160*) and its three *A. thaliana* homologs (*At4g08990*, *At4g13610*, and *At4g14140*) is altered in endosperm. In embryo, *MET1* is by far the predominantly expressed maintenance methyltransferase gene, with expression levels about 50-fold higher than those of its homologs (Table 3). This pattern is also seen in vegetative and floral tissues (43). By comparison, although none of the *MET* genes seem to be imprinted in endosperm, *MET1* is down-regulated, whereas the other three *MET* genes are up-regulated relative to their expression in embryo (Table 3). Thus, maintenance of CG methylation in endosperm is carried out by distinct *MET* and *VIM* gene family members.

Maternally Expressed Genes That Are Not Affected by Paternal *met1* and Maternal *dme* and *fie* Mutations. Among the 116 maternally expressed genes that we analyzed, we only scored 35 as affected by mutations in *met1*, *dme*, or *fie* (Fig. S1, Dataset S2, and *SI Methods*). Many of the remaining genes show significant expression in seed coat (Fig. S3*C*), raising the possibility that some of these are false positives. However, even in cDNA from CxL LCM-derived endosperm, some of these genes show clear maternal expression, including *ARGONAUTE 9* (*AGO9*; *At5g21150*), a protein kinase (*At1g29730*), and *HDG9* (Fig. S4), indicating that these genes are either imprinted or deposited from maternal tissues.

That a gene did not meet our statistical significance criteria does not necessarily prove that its imprinting is not affected by *met1*, *dme*, or *fie*. For this reason, we used a more stringent cutoff—a gene had to be expressed at least 16-fold higher from the maternal than paternal genome in *met1*, *dme*, and *fie*—to identify 19 genes that were still clearly maternally expressed in all three mutant lines (Fig. S1). These genes include *AGO3* (*At1g31290*), a MYB transcription factor gene (*At3g10590*), *ARABIDOPSIS SKP1-LIKE* E3-ligase component genes *ASK8* (*At3g21830*) and *ASK10* (*At3g21860*), and cytidine deaminase genes (*At4g29570* and *At4g29640*) (Table 4 and Dataset S2). Seed microarray data (GEO accession no. GSE12404) are available for 16 of these genes, of which seven are expressed only in chalazal seed coat and chalazal endosperm (Table 4 and Figs. S1 and S5*A*). Only 26 of 22,533 genes on the array, including the seven genes mentioned above, have this pattern of expression ($P = 3.6 \times 10^{-16}$; Fisher's exact test) (Dataset S2). This group

Table 4. Selected maternally expressed genes unaffected by *met1*, *fie*, or *dme*

Number	Annotation	CxL M/P	LxC M/P	*met1* M/P	*fie* M/P	*dme* M/P	Endo exp*	Emb exp*	*met1* exp	*fie* exp	*dme* exp
AT1G31290	AGO3	324/33	280/22	177/3	199/9	562/7	160/169	7/0	294	181	508
AT1G61090		244/0	419/2	373/2	187/1	629/0	125/127	0/0	639	161	375
AT3G10590	MYB TF	47/3	69/3	59/0	59/1	115/0	62/57	0/0	279	118	178
AT3G21830	ASK8	252/1	318/1	220/1	228/3	723/1	149/96	0/0	507	264	651
AT3G21860	ASK10	34/0	99/0	49/0	77/0	258/0	34/48	1/0	69	50	353
AT4G29570	CDA	34/4	82/5	60/1	98/1	150/0	49/75	0/0	221	119	178
AT4G29640	CDA	32/8	127/14	109/2	49/0	44/0	87/73	2/0	368	146	155

A list of maternally expressed genes, the imprinted status of which is not affected by *met1*, *fie*, or *dme*, that are expressed only in chalazal endosperm and seed coat. Total maternal (M) and paternal (P) reads are shown for the indicated genotypes as well as transcriptional scores (number of reads per kilobase of sequence per 10 million aligned reads) for endosperm (Endo exp), embryo (Emb exp), and the indicated mutant genotypes; TF, transcription factor; CDA, cytidine deaminase.
*Transcriptional scores derived from manually dissected and LCM tissue are shown before and after the slash, respectively.

includes *ASK7* (*At3g21840*) and three more cytidine deaminase genes (*At4g29580*, *At4g29600*, and *At4g29620*), which show a clear bias to maternal allele expression in our sequenced cDNA libraries (Dataset S2). The remaining genes all either show a maternal bias, have no SNPs between Col and L*er*, or had no reads detected because of the low abundance of their transcripts in endosperm (Dataset S2). Chalaza is an active site of nutrient transfer from maternal seed coat to the developing endosperm (8), suggesting that the mRNA for these genes might be synthesized in the maternal chalazal tissues and transported into the chalazal endosperm. As a control, we identified 48 genes from the microarray dataset with expression only in the chalazal seed coat (Dataset S2 and Fig. S5*B*), none of which are on our list (LCM-filtered; $P < 0.001$) of maternally expressed imprinted genes ($P = 0.0004$; Fisher's exact test) (Dataset S2), indicating that seed coat contamination is unlikely to account for our results. To further rule out contamination, we analyzed expression of four genes (*AGO3*, *MYB* transcription factor, *ASK8*, and *ASK10*) by RT-PCR in CxL LCM-derived endosperm, and all four are clearly maternally expressed (Fig. S4). A mechanism of gene imprinting that does not require MET1, FIE, or DME is also consistent with our data, but the characteristic mRNA profile makes transport much more likely.

Discussion

Our study has significantly expanded the number of known genes with parent of origin-specific expression in *A. thaliana* endosperm. We estimate (based on SNP availability and sequencing depth) that our dataset is sufficiently deep to ascertain the imprinted status of 10,755 endosperm genes or roughly one-half of the endosperm transcriptome, assuming about two-thirds of the 28,244 *A. thaliana* genes that we examined are expressed in endosperm (Fig. S1). However, taking into account our rather stringent statistical cutoff ($P < 0.001$) and filtering using LCM data, this estimate should be revised closer to 20–30% of the endosperm transcriptome. Consistent with this fraction, only 3 of 10 previously described imprinted genes (not counting *FH5*) passed all of our filters (Table S1). Thus, there may be 30–50 paternally expressed endosperm genes, about 200 maternally expressed genes regulated by DNA methylation or PcG activity, and potentially over 500 maternally biased genes if genes regulated by unknown mechanisms or deposited from maternal tissues are considered. Allele-specific gene expression is clearly a major phenomenon in plant endosperm that is comparable with the extensive imprinting recently reported in mouse brain (22).

Parental Conflict May Occur at Many Regulatory Levels. The parental conflict theory (1) proposes that nutrient allocation is the driving force for the evolution of gene imprinting in mammals and plants. Although the effect on nutrient allocation of the imprinted genes described here is not yet known, the potential lines of conflict between maternal and paternal parents have significantly expanded. At the chromatin level, in addition to the previously discovered maternally expressed PRC2, paternally expressed proteins potentially silence target genes by promoting maintenance DNA methylation (VIM5) and H3K9 methylation (SUVH7), and maternally expressed genes potentially silence targets by regulating the small RNA pathway (*DRB2*), H3K9 methylation (*SUVH8*), H3K4 demethylation, and DRM2-mediated DNA methylation (*JMJ15*). Parental conflict may occur at the posttranslational level, mediated by degradation of specific proteins through the ubiquitin-26S proteasome system, which rivals transcription as a dominant regulatory mechanism in *A. thaliana* (29). Parental conflict may also take place through protein–protein interactions. *At1g59930*, a maternally expressed imprinted gene, encodes a truncated MADS box transcription factor that lacks the MADS box domain. Although it is unlikely to bind DNA, this protein may inhibit other MADS box transcription factors through dimerization (44), including the activity of a close full-length relative, the paternally expressed PHE1. Imprinting of hormone synthesis (*YUC10* and *ACX1*) and response (*JLO* and *EIN2*) genes suggests that hormone action may also be involved in parental conflict.

Imprinted Genes Regulated by DNA Methylation Often Encode Regulatory Proteins. Imprinted expression of genes with regulatory potential is frequently regulated by DNA methylation, whereas the PRC2 complex regulates imprinting of genes that participate in cellular metabolism and signaling (Tables 1 and 2, Dataset S2, and Table S2). Polycomb group proteins function in maintaining rather than establishing the silent state (45). It is possible that DNA demethylation in the central cell initially imprints genes encoding regulatory proteins that, in turn, activate or repress other genes, the transcriptional states of which are cemented by PRC2 activity. If so, mutations in imprinted genes directly regulated by DNA methylation would be predicted to affect the transcriptional status of other imprinted genes, particularly those dependent on PRC2.

Genes Not Regulated by DME, FIE, or MET1. A subset of maternally expressed genes (*ASK*, cytidine deaminases, and *AGO3*) do not seem to be regulated by DME, FIE, or MET1. It is possible that their imprinted expression is regulated by an unknown mechanism for paternal allele silencing. Alternatively, because their mRNA is detected only in chalazal seed coat and chalazal endosperm (Fig. S5*A*), the mRNA for these genes might be synthesized in the chalazal seed coat and transported into the chalazal endosperm. If this is the case, these genes would represent an additional mechanism by which the maternal parent genetically controls seed development. There is evidence for intercellular movement of plant RNAs through plasmodesmata (46), although it remains to be experimentally tested whether RNAs can navigate the apoplastic pathways that connect the chalazal seed coat and endosperm (47). The three *ASK* genes and five cytidine deaminase genes are organized in tight clusters and likely represent recent duplication events, which might serve to coordinate this expression pattern. Such clustering was not detected among the other *A. thaliana* imprinted genes in this study, confirming that plant genes, in contrast to mammalian imprinted genes, are singletons (1). Transport of *AGO9* RNA (itself maternally biased in endosperm) (Dataset S2) from somatic companion cells mediates small RNA-dependent transposon silencing necessary for specification of gametophyte precursor cells (48), and maternally deposited *AGO3* may likewise influence transposon silencing in the endosperm. Although the significance of converting cytidine to uridine by cytoplasmic cytidine deaminase proteins in the chalazal endosperm is not clear, picomolar concentrations of uridine enhance cell division responses to auxin and cytokinin in root cortical cells (49).

Control of Genome Hypomethylation in the Endosperm by *DME*, *VIM*, and *MET* Genes. We previously showed that virtually the entire *A. thaliana* endosperm genome is demethylated in the CG context and that this demethylation is largely dependent on DME (33). Here, we show that the *VIM5* gene is primarily expressed from the paternal genome, *MET1* is down-regulated in endosperm (as has been previously shown in the female gametophyte), and *VIM* and *MET* genes are up-regulated in *dme*-deficient and *fie*-deficient endosperm (Table 3). These results suggest that CG hypomethylation in the central cell and endosperm might be orchestrated by regulation of *VIM* and *MET* genes in addition to direct DME activity. To affect CG methylation in the central cell, *VIM* and *MET* genes would have to be down-regulated before central cell differentiation to allow for

passive demethylation by DNA replication. As DME is expressed specifically in the central cell, the initial down-regulation would not require DME. However, maintenance of *VIM* and *MET* repression, which might be necessary for genome-wide hypomethylation, would require a functional PRC2 complex (Table 3), which, in turn, requires DME. Thus, one possible model would be that DME directly demethylates a number of discrete loci, whereas global demethylation is caused, at least in part, by DME-dependent *VIM* and *MET* repression. This hypothesis predicts that WT global demethylation should be dependent on PRC2 activity. This prediction is consistent with our observation that some maternally expressed genes apparently activated by removal of DNA methylation (i.e., paternal allele up-regulated by *met1* and maternal allele down-regulated by *dme*) are down-regulated in *fie* endosperm (Table 1).

Methods

Illumina cDNA libraries were constructed with the Ovation RNA-seq System (NuGen Technologies) using total RNA isolated from manually or LCM-dissected seeds 7–8 d after pollination. Each gene received Col and L*er* scores, each equal to the number of reads aligned to that gene that were assigned to the respective ecotype. Each gene also received a transcriptional score, equal to the number of reads aligned to the cDNA model (irrespective of ecotype) per 1 kb of sequence per 10 million aligned reads. We calculated the probability that a gene's expression deviates from expectation or that a gene's imprinted status is altered by mutation using Fisher's two-tailed exact test. *SI Methods* has details.

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