One-Way Control of *FWA* Imprinting in *Arabidopsis* Endosperm by DNA Methylation

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The *Arabidopsis FWA* gene was initially identified from late-flowering epigenetic mutants that show ectopic *FWA* expression associated with heritable hypomethylation of repeats around transcription starting sites. Here, we show that wild-type *FWA* displays imprinted (maternal origin–specific) expression in endosperm. The *FWA* imprint depends on the maintenance DNA methyltransferase MET1, as is the case in mammals. Unlike mammals, however, the *FWA* imprint is not established by allele-specific de novo methylation. It is established by maternal gametophyte–specific gene activation, which depends on a DNA glycosylase gene, *DEMETER*. Because endosperm does not contribute to the next generation, the activated *FWA* gene need not be silenced again. Double fertilization enables plants to use such "one-way" control of imprinting and DNA methylation in endosperm.

DNA methylation is a key epigenetic determinant that controls parent of origin–specific gene expression (imprinting) in mammals, where the methylation is erased and reestablished in each generation (*1*). In contrast, epigenetic states of gene expression in flowering plants are often inherited unchanged over many generations. Epigenetic mutations affecting plant development have been identified in laboratories and in natural populations (*2*–*9*). For example, the *Arabidopsis* late-flowering mutant *fwa-1* does not have a change in the nucleotide sequence of the responsible gene *FWA*; instead, the phenotype is due to ectopic *FWA* expression associated with heritable loss of methylation (*5*). Although the loss of DNA methylation induces the late-flowering phenotype, there has been no evidence that *FWA* methylation is developmentally regulated in the wild type. Nor does this gene seem to control flowering time during normal development, because loss-of-function mutations of *FWA* do not affect flowering time (*5*). To understand the role of DNA methylation in plant development, we examined the expression of *FWA* during normal development.

FWA is not expressed in wild-type adult tissues, but the *FWA* transcripts are detectable in the silique and in 4-day imbibed seeds (*5*). We first examined *FWA* expression by reverse transcription polymerase chain reaction (RT-PCR) in various organs and dissected seeds (Fig. 1A). In the dissected seeds, *FWA* transcripts were not detected in the embryo fraction but were detected in the fraction containing endosperm and the seed coat. Examination of other organs suggests that *FWA* expression is confined to the developing endosperm or seed coat.

We monitored *FWA* expression in plants with a *pFWA::FWA-GFP* transgene that express an FWA–green fluorescent protein (GFP) fusion protein under the control of the *FWA* promoter. Using confocal laser microscopy, we localized the FWA-GFP fusion protein to the central cell nucleus before fertilization, which is the progenitor of endosperm in the mature ovule (Fig. 1B). After fertilization, GFP fluorescence was observed in the fertilized central cell and the developing endosperm up to the 8- to 16-nuclei stage (Fig. 1, C to F). GFP fluorescence was not detected in the egg cell or embryo in these two constructs. We also confirmed endosperm-specific expression of *FWA* by in situ hybridization (fig. S1, G and H). Taken together, these results suggest that *FWA* expression is confined to the central cell of the female gametophyte and the endosperm. The transcripts were not detected in the embryo or vegetative organs.

Ectopic expression of the *FWA* gene in the *fwa-1* hypomethylated epigenetic allele is accompanied by the loss of DNA methylation of the direct repeats of the 5′ region of the gene (*5*). To determine whether endosperm-specific expression is also correlated with loss of DNA methylation, we examined *FWA* methylation in various seed tissues and in pollen by the bisulfite sequencing procedure. We dissected the seed into three parts—embryo, seed coat, and endosperm (fig. S2)—and isolated DNA from each part. The

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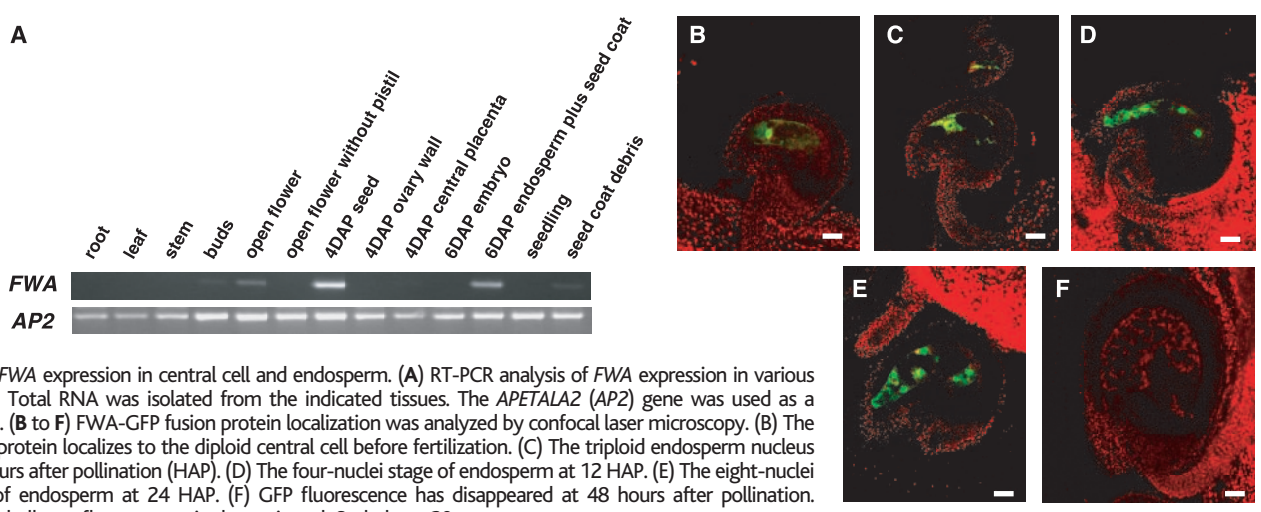

Fig. 1. *FWA* expression in central cell and endosperm. (**A**) RT-PCR analysis of *FWA* expression in various organs. Total RNA was isolated from the indicated tissues. The *APETALA2* (*AP2*) gene was used as a control. (**B** to **F**) FWA-GFP fusion protein localization was analyzed by confocal laser microscopy. (B) The fusion protein localizes to the diploid central cell before fertilization. (C) The triploid endosperm nucleus at 6 hours after pollination (HAP). (D) The four-nuclei stage of endosperm at 12 HAP. (E) The eight-nuclei stage of endosperm at 24 HAP. (F) GFP fluorescence has disappeared at 48 hours after pollination. Chlorophyll autofluorescence is shown in red. Scale bars, 20 μm.

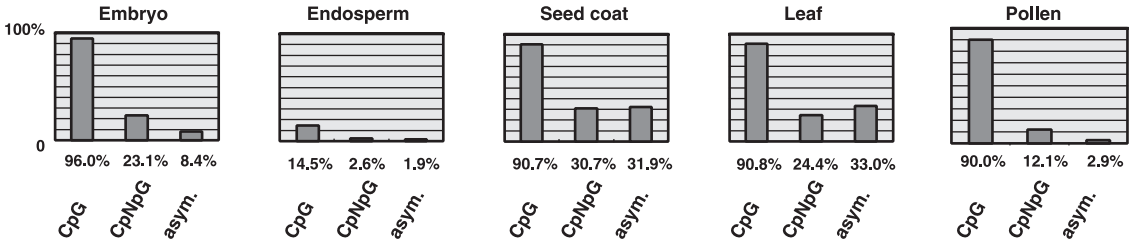

Fig. 2. Demethylation of *FWA* in endosperm. Percent methylation at CpG, CpNpG, and asymmetric sites of the 5′ direct repeats of the *FWA* gene was determined by bisulfite sequencing with individual 6-12 clones. DNA from each tissue was isolated from Col-0 accession.

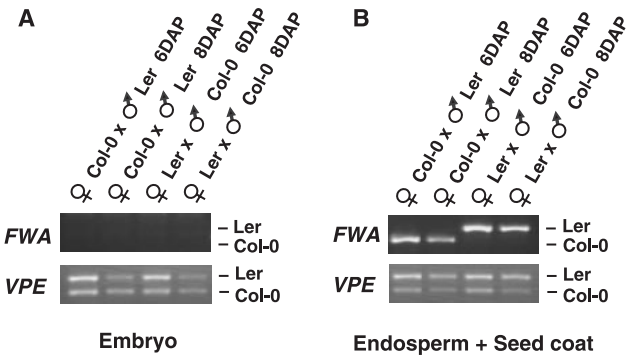

Fig. 3. Imprinting of *FWA* in endosperm. (**A**) Allele-specific RT-PCR shows no *FWA* expression in embryo. (**B**) Maternal allele–specific expression in endosperm at 6 and 8 days after pollination (DAP), corresponding to torpedo and early maturation stages of embryo development. The nonimprinted α*VPE* gene was used as a control.

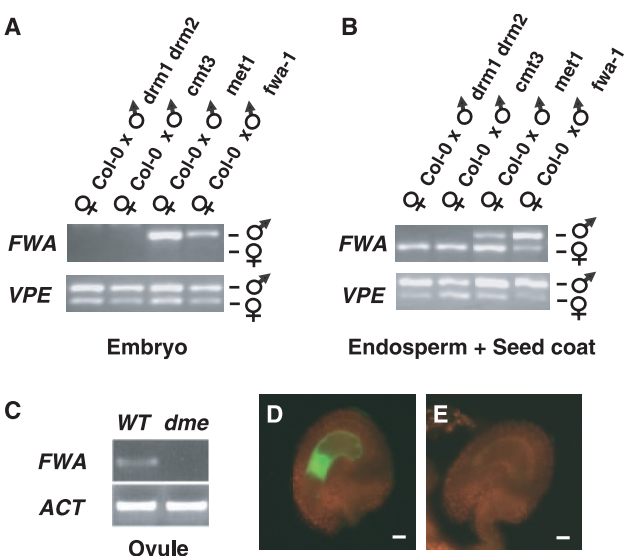

Fig. 4. Trans mutations affecting *FWA* imprinting. (**A** and **B**) Col-0 females were crossed with *drm1 drm2* (Ws), *cmt3-7* (L*er*), *met1-1* (L*er*), or *fwa-1* (L*er*) mutants. The polymorphic site of Ws is the same as for L*er*. Total RNA was isolated from dissected embryo and endosperm plus seed coat fractions at 7 days after pollination (corresponding to the walking-stick stage of embryo development) and was subjected to the allele-specific RT-PCR analysis. (**C**) RT-PCR analysis of *FWA* transcripts in wild type and *dme-1* mutant. The *ACTIN* (*ACT*) gene was used as a control. (**D** and **E**) Fluorescence images of *pFWA::FWA-GFP* expression in wild type (D) and *dme-1* homozygous mutant (E). Scale bars, 20 μm.

overall DNA methylation level in the *FWA* 5′ direct repeats was markedly reduced in the endosperm (Fig. 2). By contrast, *FWA* methylation was not reduced in the embryo, seed coat, leaf, and pollen (Fig. 2).

Endosperm is the only tissue in which parental imprinting has been reported in flowering plants (*10*–*14*). We used allele-specific RT-PCR analysis to test for *FWA* imprinting. A C/A polymorphism between strains Col-0 and L*er* in exon 7 was used to distinguish the transcripts from the maternal and paternal *FWA* alleles. In F_1 seeds resulting from reciprocal interstrain crosses, only the transcripts derived from the maternal allele were detected in the endosperm plus seed coat fractions, whereas both the maternal and paternal alleles were silent in the embryo (Fig. 3). The *FWA* imprinting in endosperm was also confirmed in other interstrain crosses between strains Col-0 and Ws (fig. S3A).

We next examined whether the tissue-specific and parent of origin–specific *FWA* expression depends on DNA methylation. Wild-type Col-0 was reciprocally crossed to mutants of DNA methyltransferases (Fig. 4, A and B) (fig. S3B) and the effect of each mutation on *FWA* expression was examined by allele-specific RT-PCR using dissected F_1 seeds. Paternally derived *FWA* transcripts were detected when the male parent had a mutation in *MET1*, the maintenance methyltransferase for CpG sites (*15*, *16*) (Fig. 4, A and B). The *met1* mutation in the female parent did not induce paternal *FWA* expression in the embryo or endosperm plus seed coat fractions (fig. S3B), which suggests that the loss of paternal silencing occurred before fertilization. On the other hand, the imprinting was not affected by mutation of *CMT3* (*CHROMOMETHYLASE3*) or by mutation of the *DRM* (*DOMAINS REARRANGED METHYLTRANSFERASE*) de novo methylase (Fig. 4, A and B) (fig. S3B). CMT3 has been shown to be important for methylation of non-CG sites (*17*, *18*). DRMs are structurally similar to mammalian Dnmt3 de novo methylases (*19*, *20*), and *DRM2* is necessary for the de novo methylation induced by transgenes (*21*). The *met1* mutation also induces *FWA* expression in the embryo (Fig. 4A). These results suggest that maintenance of endosperm-specific and parent of origin–specific *FWA* expression depends on *MET1*.

Because the DRM de novo methyltransferases did not affect *FWA* imprinting, a remaining important question is how the specific DNA methylation and expression patterns are established in the endosperm. We next examined the effect of *DME*, which has been shown to activate expression of the maternal *MEDEA* (*MEA*) allele in central cells before fertilization (*22*). *DME* encodes a protein with a DNA glycosylase domain, and the product has a DNA glycosylase activity in vivo. We tested the effect of the *dme-1* mutation on expression of *FWA*. RT-PCR revealed that *FWA* transcripts did not accumulate in homozygous *dme-1* mutant ovules, whereas they were detectable in control wild-type ovules (Fig. 4C). We also examined the effect of the *dme-1* mutation on *FWA* promoter activity. A *pFWA::FWA-GFP* transgenic line was crossed to *dme-1* mutant plants. In the F_2 progeny from this cross, GFP fluorescence was detected in the central cell nucleus of wild-type *DME* ovules (Fig. 4D), but no signal was observed in homozygous mutant *dme-1* ovules (Fig. 4E). These results suggest that the maternal-specific *pFWA::FWA-GFP* expression depends on a functional *DME* allele in the female gametophyte. Although no *DME* product is detectable after fertilization (*22*), its effect on *FWA* expression is prolonged after fertilization, which suggests that DME affects a heritable epigenetic mark on *FWA*, as is the case for *MEA* (*22*).

Our results indicate that the maintenance of *FWA* imprinting depends on the

maintenance DNA methylation machinery, a situation comparable to mammalian imprinting (*23*). Unlike mammals, however, the maternal-specific expression of *FWA* is not established by a paternal-specific de novo methylation, but it is established by maternal-specific activation that is dependent on the *DME* DNA glycosylase. Thus, the silent methylated state is the default for this class of imprinted genes (*22*). It would be important to know how general such a controlling mechanism is in plants. The control by *DME* is conserved between *FWA* and *MEA*. However, it has been reported that loss of *MET1* activity with a paternally transmitted transgene with *MEA* promoter does not induce its activation (*14*). On the other hand, *MET1* regulates *MEA* expression in the female gametophyte in an antagonistic manner to *DME* (*24*). Thus, control of imprinting by MET1 and DME might be a general mechanism. In any case, a unique feature of imprinting in flowering plants is that the epigenetic state in the endosperm does not need to be reprogrammed again. Because the endosperm degenerates during seed maturation, it does not transmit genetic or epigenetic information to the next generation. In this sense, the endosperm is functionally analogous to mammalian extra embryonic membrane. Establishment of imprinting in the central cell and its subsequent maintenance in endosperm after double fertilization enables plants to use such simple one-way control of imprinting. When methylation is lost in the embryonic lineages (e.g., by the *met1* mutation), the *fwa* epigenetic mutation and its associated late-flowering phenotype can be stably inherited over many generations.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1089835/DC1
Materials and Methods
Figs. S1 to S3
References

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Editing of CD1d-Bound Lipid Antigens by Endosomal Lipid Transfer Proteins

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It is now established that CD1 molecules present lipid antigens to T cells, although it is not clear how the exchange of lipids between membrane compartments and the CD1 binding groove is assisted. We report that mice deficient in prosaposin, the precursor to a family of endosomal lipid transfer proteins (LTP), exhibit specific defects in CD1d-mediated antigen presentation and lack Vα14 NKT cells. In vitro, saposins extracted monomeric lipids from membranes and from CD1, thereby promoting the loading as well as the editing of lipids on CD1. Transient complexes between CD1, lipid, and LTP suggested a "tug-of-war" model in which lipid exchange between CD1 and LTP is on the basis of their respective affinities for lipids. LTPs constitute a previously unknown link between lipid metabolism and immunity and are likely to exert a profound influence on the repertoire of self, tumor, and microbial lipid antigens.

CD1 molecules have evolved a unique hydrophobic binding groove that binds lipid antigens in both the secretory and endosomal compartments for presentation to T lymphocytes (*1*). In mice, the main population of CD1-restricted T cells, called Vα14 NKT cells, express a semi-invariant Vα14-Jα18/Vβ8 T cell receptor (TCR). These cells exhibit reactivity against CD1d in combination with endogenous ligands (*2*) that can be mimicked by α-galactosylceramide (αGC) (*3*). This population is conserved among mammalian species and regulates immune responses (*4*, *5*). Like human CD1b-restricted T cells specific for mycobacterial glycolipids (*6*), Vα14 NKT cells are dependent on endosomal trafficking of CD1d for natural antigen recognition (*7–10*). Other endogenous or exogenous antigens do not require endosomal trafficking, however, suggesting that loading may be achieved in distinct cellular compartments depending on the nature of the antigen (*9*, *11*, *12*). CD1 endosomal trafficking is tightly controlled by cytoplasmic tail–encoded tyrosine-containing motifs binding adaptor protein 2 and 3 (AP-2 and AP-3) complexes, as well as by association with the invariant chain (Ii) or Ii/major histocompatibility (MHC) class II complexes (*13–16*).

Because lipids are integral membrane components that might require lipid transfer proteins (LTP) (*17*) for extraction, we investigated whether various families of LTP might assist antigen presentation. We focused

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