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

Tetsu Kinoshita, Asuka Miura, Yeonhee Choi, Yuki Kinoshita, Xiaofeng Cao, Steven E. Jacobsen, Robert L. Fischer, Tetsuji Kakutani

Supplementary Online Material

Materials and Methods

Plant materials and growth conditions. Plants were grown on vermiculite with nutrient supplements of Hyponex (1:2000 dilution, Hyponex Inc. Japan) under long day condition (16 hr; light, 8 hr; Dark) at 22 C°. *fwa-1* was obtained from ABRC Stock center.

RNA isolation and allele-specific RT-PCR. Col-0, WS, L*er*, *drm1 drm2* (WS), *cmt3-7* (L*er*), *met1-1* (backcrossed six times to L*er*) , *fwa-1* (L*er*) were used for crossing. F_1 seed dissection was performed as described (*1*). Total RNA was isolated from tissues using RNAeasy plant mini kit (Qiagen). First strand cDNA was synthesized using Retroscript kit (Ambion) with gene specific primers of *FWA-RTr1*: 5'-CTTTGGTACCAGCGGAGA-3', and *AP2-RTr1*: 5'-GTTCGCCTAAGTTAACAAGAGGA-3', and *αVPE-RTr1*: 5'-CCAATCGTCAACAAGCGGT-3', respectively. RT-PCR analysis were carried out with following nested primers: *FWA-dNheI*: 5'-GCCACTTTTGGTTCCACCAGAACCGGTAGCTA-3' and *FWA-RTf*: 5'-GATCCAAAGGAGTATCAAAGATCT-3', *AP2-RTr2*: 5-GAGGAGAGAATCCTGATGATGCT-3' and *AP2-RTf*: 5'-GCTTAACCATACTCCCAATTCA-3'. Allele specific RT-PCR was performed as described (*1*).

Bisulfite genomic DNA sequencing. Bisulfite sequencing was performed as described (*2*). The PCR amplified fragment of the bisulfite treated DNA was gel purified and cloned into pT7Blue plasmid (Novagene) and 6 – 12 independent clones were sequenced. For the positive control of bisulfite chemical reaction, unmethylated *ASA1* gene (*3*) and *fwa-1* allele were used.

Plasmid construction and transgenic plants. To create *pFWA::FWA-GFP* (Fig. 1) and *pFWA::ΔFWA -GFP* (Fig. S1) constructs, the BAC clone (F11K11) was amplified with the primers *TKF1*: 5'- AATTCAAGCTTGGTAGGCTAATAATCAGAAGCCCT-3' and *TKR4*: 5'-ACCATGGATCCGCCACCACCGCCACCCGTCTGTAAATCTAACGCGGATTTGA-3', and the primers *TKF1* and *TKR2*: 5'-ACCATGGATCCACCACCGCCCTTCTCGAGATTTCTTTTATTCTGGAACCA, respectively. The PCR fragments were digested with *Hind* III and *Nco* I and ligated to the *sGFP* plasmid vector, respectively (*4*). *pFWA::FWA-GFP* construct contains the promoter sequence 3274bp upstream from first ATG codon) and the entire sequence of coding region of the *FWA* gene, and replaced stop codon with glycine linker sequence and the translational fusion with the *GFP* reporter. *pFWA::ΔFWA -GFP* construct contains the promoter sequence, which is identical to *pFWA::FWA-GFP*, and homeodomain and nuclear localization signal of the FWA protein. Plants were transformed via *Agrobacterium EHA101* as described (*5*).

Histology and Microscopy. Tissue fixation, sectioning and *in-situ* hybridization was performed as described procedure (*6*). Digoxigenin labeled RNA probe was synthesized with in vitro transcription using T7 RNA polymerase according to manufacture's instruction (Boehringer Manheim). Sense and antisense probes were prepared from plasmid that contain 1459 bp of 3' region of the *FWA* cDNA. Bright field and fluorescence images were taken with a Zeiss Axioplan 2 microscope equipped with Axiovision 3.1 system. Optic sections were made with Olympus FV500 confocal laser microscope.

Supporting Figures

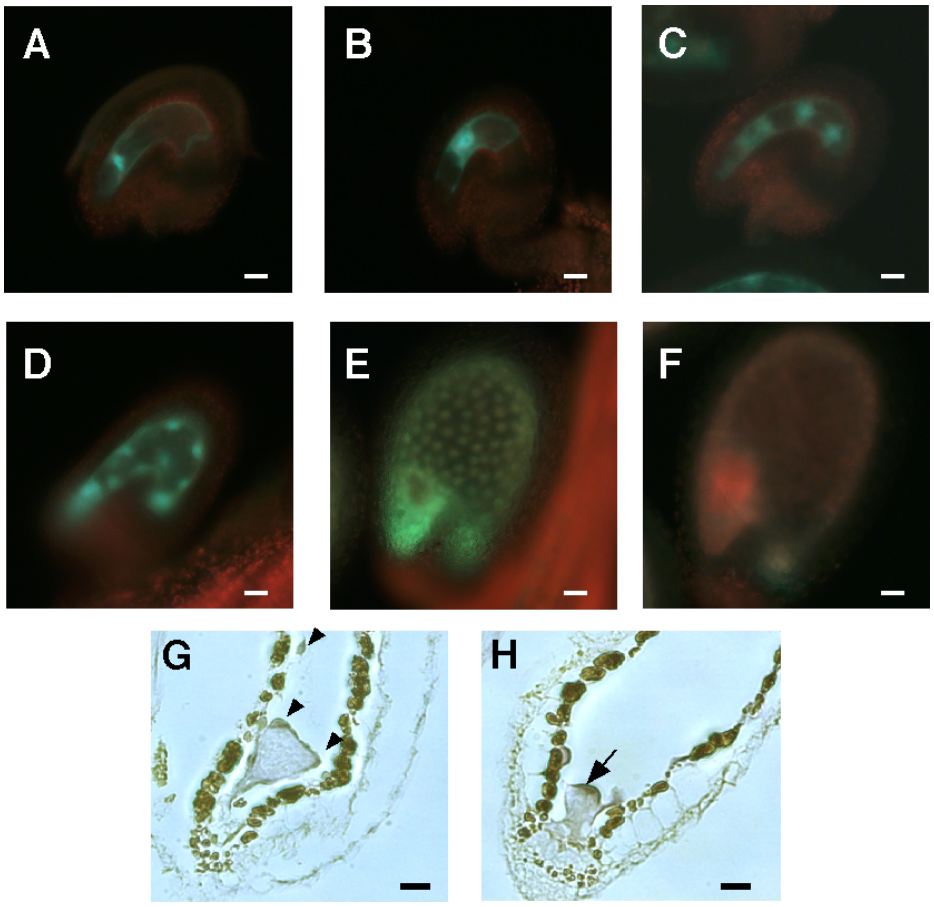

Fig. S1. Expression pattern of the *FWA* gene. (A to F) Fluorescence images of the *pFWA::ΔFWA-GFP* transgenic plants. Unlike *pFWA::FWA-GFP* fusion construct in Figure 1, *ΔFWA-GFP* expression has prolonged expression pattern; GFP is visible in mature central cell nucleus before fertilization (A), primary endosperm nucleus after fertilization (B), 4 nuclei endosperm stage (C), 16-32 nuclei endosperm stage (D), micropylar endosperm (an arrow with dashed line), peripheral endosperm nuclei (arrowheads), chalazal endosperm (an arrow with solid line), in the heart stage of embryo development corresponding to 5 days after pollination (E), endosperm in chalazal end in the torpedo stage of embryo development corresponding to 6 days after pollination (F) (The anatomy of endosperm development in Arabidopsis is described in ref. *7*). The discrepancy of two constructs may

reflect post-translational modification of the FWA protein (i.e. FWA-GFP has whole part of the FWA protein, while ΔFWA-GFP has N-terminal homeodomain only). (G and H) *In situ* hybridization showing *FWA* transcripts. *FWA* transcripts were observed in the free-nuclear endosperm (arrowheads in G), and in the cyst of chalazal endosperm at the heart stage of embryo development (an arrow in H; comparable to an arrow with solid line in E). Brown colors at the inner layer of the seed coat were an artifact of paraformaldehyde fixation. Bars = 20 μm (A to D), 40 μm (E to H).

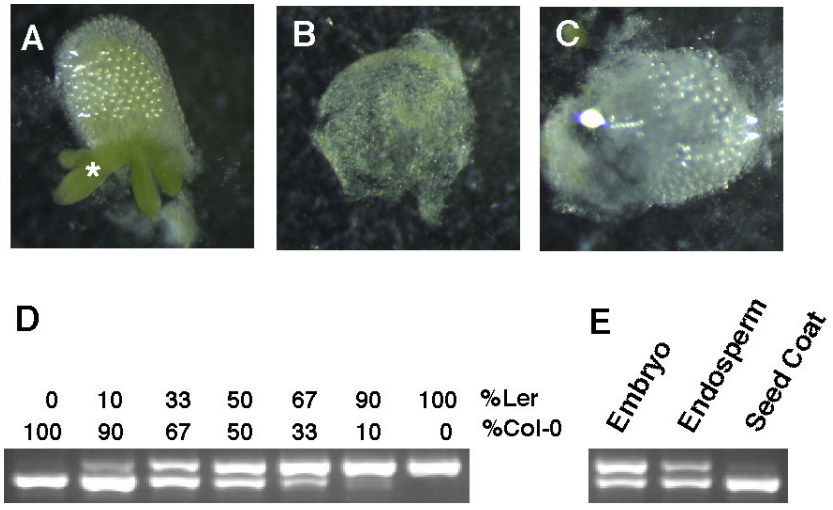

Fig. S2. Origins of the dissected seed fractions. (A to C) Pictures of dissected tissues. A walking stick stage embryo (asterisk) (A). An endosperm tissue (B) separated from seed coat (C). (D and E) Quality estimation of the seed dissection procedure. (D) Col-0 and L*er* genomic DNA were mixed as indicated above the panel, and subjected to allele specific PCR analysis. C/A polymorphism in exon 7 of the *FWA* gene was used. Upper bands represent L*er* genomic DNA, while lower bands are Col-0. (E) Col-0 and L*er* accessions were crossed, and then the F_1 seeds were dissected into the embryo, endosperm and seed coat, respectively. The genomic DNA was isolated from those tissues and analyzed by allele-specific PCR to measure the amount of the maternal and paternal DNA. The band

intensity of the embryo, endosperm, and seed coat fractions agreed well with the expected maternal: paternal ratio (i.e. 1:1 in the embryo, 2:1 in the endosperm, and 2:0 in the seed coat).

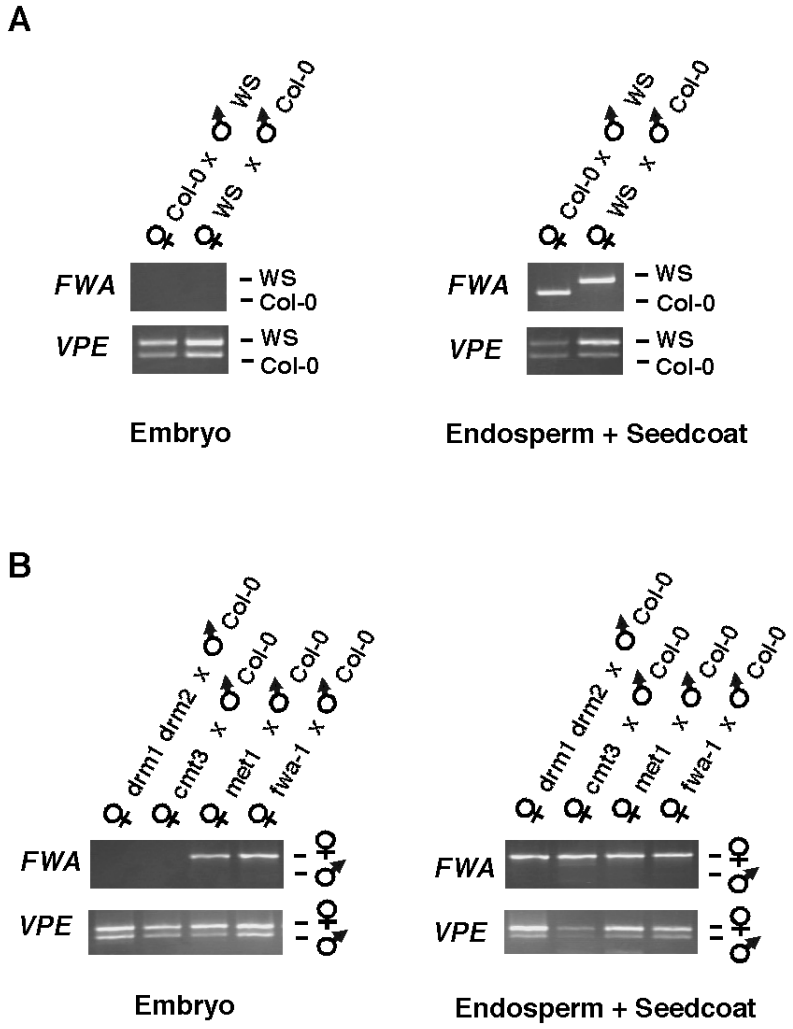

Fig. S3. RT-PCR results supplementing those in Fig. 4 (A) FWA imprinting examined in F_1 hybrids between WS and Col. The WS allele can be distinguished from the Col allele in the same polymorphic site used to distinguish L*er* from Col alleles (not shown). The *FWA* allele showed imprinted expression at 7 days after pollination in the endosperm plus seed coat fractions in this cross as is the case for crosses between L*er* and Col. (B) Results from

crosses reciprocal to those shown in Fig 4. The mutants *drm1 drm2* (WS), *cmt3-7* (L*er*), *met1-1* (L*er*), *fwa-1* (L*er*) were crossed with male wild type Col-0. In endosperm, none of the mutants affected suppression in the paternal allele and activation in the maternal allele. Monoallelic *FWA* transcript was detected in embryo when *met1-1* or *fwa-1* mutant was used for the female parent (left panel) as is the case when those mutants were used for the male parents (Fig. 4).

Supporting References

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3. J. A. Jeddeloh, J. Bender, E. J. Richards, *Genes Dev.* 12, 1714 (1998).
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6. D. P. Jackson, *In situ hybridization in plants*. D. J. Bowles, S. J. Gurr, M. McPhereson, Eds. *Molecular plant pathology. A practical approach* (Oxford University Press, Oxford, UK., 1991).
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