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Polycomb group-dependent imprinting of the actin regulator *AtFH5* regulates morphogenesis in *Arabidopsis thaliana*

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During embryogenesis, Polycomb group (PcG) complexes deposit silencing histone modifications and target homeotic genes, which regulate the patterning of other transcription factors. This transcriptional network further maintains cell fate. However, genome-wide identification of histone modifications has suggested that PcG complexes might regulate genes other than those encoding transcription factors. In *Arabidopsis*, we show that PcG activity directly targets the actin regulator formin *ARABIDOPSIS FORMIN HOMOLOGUE 5* (*AtFH5*). PcG activity silences the paternal allele of *AtFH5*, restricting its expression to the maternal allele. *AtFH5* thus appears to be a new, maternally expressed imprinted gene. We further demonstrate that *AtFH5* is responsible for morphological defects caused by the loss of PcG activity in the seed.

KEY WORDS: Polycomb, Endosperm, *Arabidopsis*, Imprinting, *ARABIDOPSIS FORMIN HOMOLOGUE 5* (*AtFH5*)

INTRODUCTION

Parental genomic imprinting – the preferential expression of one of the two parental alleles of a gene – has been described in flowering plants and mammals (Feil and Berger, 2007). In plants, distinct fertilizations involving two pairs of male and female gametes produce the plant embryo and the endosperm. The endosperm controls the supply of maternal nutrients to the embryo (Berger et al., 2008). The endosperm is the only tissue for which imprinted genes have been identified in plants (Kinoshita et al., 2008). In maize, only maternally expressed imprinted genes have been identified (Gutierrez-Marcos et al., 2004; Gutierrez-Marcos et al., 2006; Hermon et al., 2007; Kermicle, 1970). In *Arabidopsis*, among the five imprinted genes identified, *PHERES1* (*PHE1*) is paternally expressed (Makarevich et al., 2006), whereas *MEDEA* (*MEA*) (Kinoshita et al., 1999), *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) (Jullien et al., 2006b), *FLOWERING WAGENINGEN* (*FWA*) (Kinoshita et al., 2004) and *MATERNALLY EXPRESSED PAB-C TERMINAL* (*MPC*) (Tiwari et al., 2008) are expressed maternally. DNA methylation of cis-elements in the 5′ control region is responsible for the silencing of *MPC*, *FWA* and *FIS2*. By contrast, methylation of histone H3 lysine 27 (H3K27) residues is essential for silencing the non-expressed allele of *MEA* and *PHE1* (Gehring et al., 2006; Jullien et al., 2006a; Makarevich et al., 2006), although the imprint of *PHE1* further requires DNA methylation at 3′ sites (Makarevich et al., 2008). *MEA* and *FIS2* are core members of the endosperm-specific FERTILIZATION INDEPENDENT SEED (FIS) Polycomb group (PcG) complex that also includes *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*) (Guitton and Berger, 2005).

The wild-type endosperm posterior pole (also called the chalazal pole) is distinguished from the peripheral and anterior (micropylar) domains of the endosperm by a multinucleate structure termed the cyst (Boisnard-Lorig et al., 2001; Brown et al., 1999; Scott et al., 1998). The cyst develops from the migration of nuclei from the peripheral endosperm (Guitton et al., 2004). Early endosperm syncytial development ends when cellularization partitions the syncytium into mononucleate cells, but cellularization does not occur in the posterior pole (Brown et al., 1999; Sorensen et al., 2002). The endosperm of *fis* mutants is characterized by multiple defects, including enhanced proliferation, much enlarged posterior structures and the absence of cellularization (Guitton et al., 2004; Kiyosue et al., 1999; Kohler et al., 2003; Luo et al., 1999). This pleiotropic phenotype might be the consequence of the maintenance of a juvenile developmental program (Ingouff et al., 2005b). Characterized targets of the FIS PcG complex are transcriptional regulators (Makarevich et al., 2006). The pathways downstream of this transcriptional regulation are unknown and the targets, the functions of which explain the *fis* mutant phenotype, have not been identified.

We have previously characterized *ARABIDOPSIS FORMIN HOMOLOGUE 5* (*AtFH5*), which is expressed in the endosperm (Ingouff et al., 2005a). Formins are actin-nucleating agents that are involved in cell polarity and cytokinesis throughout eukaryotes (Kovar, 2006; Wallar and Alberts, 2003). Insertional mutants, *atfh5-1* and *atfh5-2*, are defective in endosperm posterior pole structures (Ingouff et al., 2005a). We report that *AtFH5* is a new imprinted gene. The FIS PcG activity restricts expression of *AtFH5* to the maternal allele and in the posterior endosperm. We identify a cis-element that is targeted by PcG activity and responsible for *AtFH5* imprinting. We further study the genetic interaction between *FIS* genes and *AtFH5* and assess the relationships between PcG-mediated regulation of *AtFH5* and endosperm morphogenesis.

MATERIALS AND METHODS

Arabidopsis strains

Seeds of all ecotypes were obtained from the *Arabidopsis* Biological Resource Center (www.Arabidopsis.org). *atfh5* mutants (Ingouff et al., 2005a) and the *fis* PcG mutants *mea-6* and *fis2-6* (Boisnard-Lorig et al., 2001; Guitton et al., 2004) were described previously.

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Microscopy
Seeds were imaged using DIC optics and Feulgen staining as reported previously (Boisnard-Lorig et al., 2001; Guitton et al., 2004), using a Leica DM600 microscope for DIC optics and a Zeiss LSM 510 META system (Zeiss, Jena, Germany; 40× Plan objective).

Expression studies
RNA extraction and treatments were as described previously (Ingouff et al., 2005a). For quantitative PCR, tissues were collected from dissected seedlings 10 days after planting on MS-Agar (Sigma). SYBR Green Master Mix was used according to the manufacturer's instructions in a 7900HT Fast Real-Time PCR apparatus (Applied Biosystems, Carlsbad, CA, USA)

Chromatin immunoprecipitation (ChIP)
Extraction of chromatin from seeds 4-5 days after pollination was conducted using a ChIP Assay Kit (Upstate, Lake Placid, NY, USA) according to the manufacturer's instructions, using antibodies against di- and trimethylated histone H3 (Lys27) (Upstate, Cornell, Ithaca, NY, USA). To detect pull-down of the *AtFH5* promoter, semi-quantitative PCR reactions were employed, with 25 cycles at an annealing temperature of 58°C (55°C for *ACT11*) and using Illustra Taq polymerase (GE Healthcare, Chalfont St Giles, UK).

Construction of vectors
Isolated *H2B-mRFP* PCR product including a 35S terminator was fused to the indicated *AtFH5* promoter regions and 5′UTR by marker-fusion PCR (Kitazono et al., 2002) then recombined with Alligator2 (Bensmihen et al., 2004) by Gateway cloning (Invitrogen, Carlsbad, CA, USA). Alligator2 was first digested with *Hin*dIII and *Eco*RV to remove the 2×35S promoter, ATG and 3×HA tag.

RESULTS AND DISCUSSION
Imprinting of *AtFH5* and its regulation by PcG activity
We fused the endogenous *Arabidopsis thaliana AtFH5* promoter to a construct encoding the nuclear reporter histone H2B-red fluorescent protein (*pAtFH5::H2B-mRFP*) to study the expression of *AtFH5* in the gametes prior to fertilization. We could not detect expression of *pAtFH5::H2B-mRFP* in the female gametes (Fig. 1A). *pAtFH5::H2B-mRFP* expression in the pollen was limited to the vegetative nucleus, and was not observed in the two male gametes (Fig. 1B). From these observations, we concluded that *AtFH5* is not expressed prior to fertilization in either the male or female gametes.

AtFH5 is expressed only in the endosperm during seed development and not in the embryo nor in the surrounding maternal seed integuments (Ingouff et al., 2005a). Hence, we were able to study the parental origin of the expression of *AtFH5* in endosperm by RT-PCR using isolated seeds. We identified a sequence polymorphism between the Landsberg *erecta* (L*er*) and C24 *Arabidopsis* accessions (Fig. 1C). If *AtFH5* were expressed from both the maternal and paternal alleles, we would have detected *AtFH5* mRNA from either accession. However, we detected only transcripts from the maternal allele of *AtFH5* in crosses between L*er* and C24 parents (Fig. 1C). Since *AtFH5* was not expressed in the central cell prior to fertilization, we conclude that the *AtFH5* transcripts we detected do not originate from the central cell and that *AtFH5* is a maternally expressed imprinted gene.

Expression of the paternal allele of *AtFH5* was regained by maternal loss of PcG function in the endosperm (Fig. 1C). This result suggested that the maternal FIS PcG complex maintains silencing of the paternal allele of *AtFH5*. Other PcG imprinted targets, *MEA* and *PHE1*, are suppressed by PcG complexes active in vegetative tissues (Jullien et al., 2006a; Katz et al., 2004; Makarevich et al., 2006; Schubert et al., 2006). In accordance,

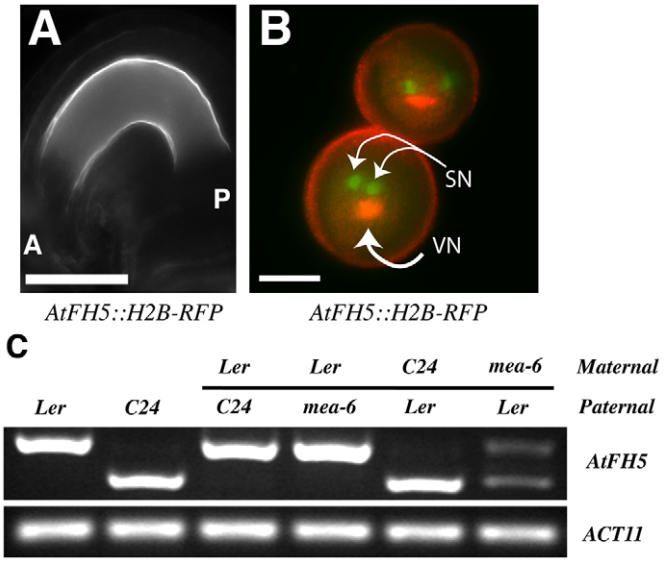

Fig. 1. *MEDEA* maintains the paternal imprint of *AtFH5*. The indicated reporter lines were compared for nuclear RFP expression in gametes. (**A**) Ovules were examined prior to fertilization. In contrast to *ACT11::H2B-mRFP* (not shown), no red fluorescent nuclei were detected in unfertilized ovules (*n*>100 seeds from each of four independent lines). (**B**) Pollen was stained with DAPI (green channel) and pollen cell nuclei examined for RFP fluorescence (red). RFP was restricted to the vegetative nucleus of pollen (VN) in all transgenic lines tested. RFP was not detected in the two sperm nuclei (SN) that fertilize the egg cell and central cell. (**C**) RNA was extracted from siliques of the indicated genotypes 5 days after pollination. An *AtFH5* sequence polymorphism between the Landsberg *erecta* (L*er*) and C24 *Arabidopsis* accessions results in a larger restriction fragment after *Eco*RI digestion of the RT-PCR products from wild-type L*er*. *ACT11* is used as a loading control. *mea-6* is in the C24 background. A, anterior pole; P, posterior pole. Scale bars: 50 μm in A; 10 μm in B.

compromised PcG activity caused by co-suppression of *FIE* or by loss-of-function of the PcG gene *SWINGER* (*SWN*) caused a significant increase of *AtFH5* expression. In the sporophyte, *AtFH5* expression was detectable in the roots, but not in above-ground tissues (Fig. 2B; see Fig. S1 in the supplementary material). Loss of *FIE* or *SWN* resulted in an up-to-fourfold increase in *AtFH5* expression in the shoot, as compared with that in the root, where the PcG genes were not repressed (Fig. 2C).

To assess whether the PcG-dependent histone methylation on lysine residue 27 was deposited on the *AtFH5* promoter, we performed a chromatin immunoprecipitation (ChIP) analysis to detect di- and trimethylation at H3K27 (H3K27me2,3). H3K27 methylation was detected within a 342 bp region of the *AtFH5* promoter, but not in the coding region (Fig. 2C). From the above results, we concluded that PcG complexes are likely to deposit marks on the promoter of *AtFH5* that are involved in the repression of its transcription in vegetative tissues and gametes.

Identification of a cis-element for PcG regulation of *AtFH5* expression
To assess the function of the region marked by H3K27me2,3 in the *AtFH5* promoter we constructed transgenic plant lines expressing a series of H2B-mRFP reporters driven by sequences derived from the *AtFH5* promoter (Fig. 2A). We identified the region *CUT4*, which includes a 417 bp region around the site marked by H3K27me2,3

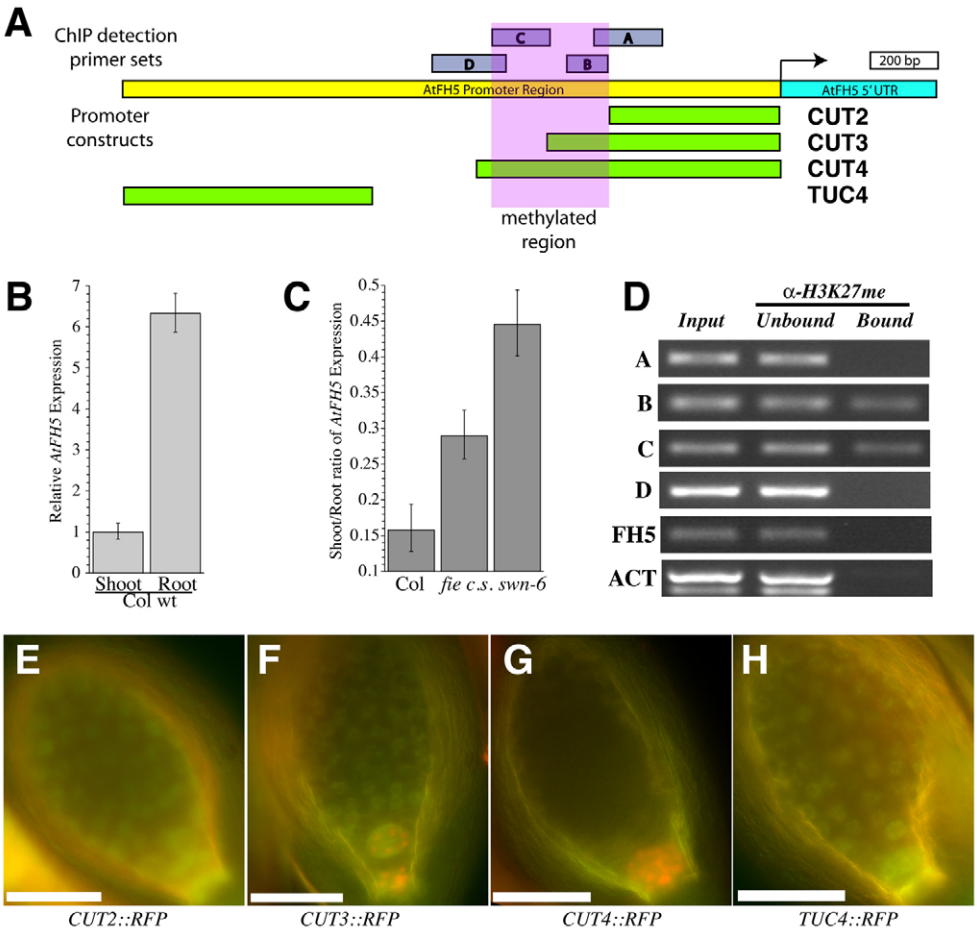

Fig. 2. FIS PcG response element in the *AtFH5* promoter. (**A**) Scale representation of the *AtFH5* promoter indicating the regions used for detection in ChIP analysis (top, blue bars) and the promoter regions fused to *H2B-mRFP* (bottom, green bars). Violet shading indicates the 342 bp region delimited by the 'B' and 'C' PCR products, where methylated histone was identified. (**B**,**C**) The relative expression of *AtFH5* based on wild-type levels in at least three biological replicates. Error bars represent Relative Quantification Min and Max values. (**D**) Fixed chromatin from young wild-type seedlings was purified then immunoprecipitated with antibodies against histone H3 methylated at lysine residue 27 (H3K27me). PCR was used to detect *AtFH5* sequence in the antibody-bound fraction, as compared with both unbound and input chromatin. (**E-H**) Expression of *AtFH5* promoter constructs in endosperm. These sequences either contained (*CUT4*), or bisected (*CUT3*), or excluded (*TUC4* and *CUT2*) a 417 bp region around the site marked by H3K27me enrichment (see A). Each transgenic line expressed H2B-mRFP in the pollen vegetative cell, similar to Fig. 1B, demonstrating transcriptional competence (data not shown). In the endosperm, however, only the reporter containing the *CUT4* promoter reproduced the wild-type pattern of *AtFH5* expression (G, 19 independent transgenic lines). The *CUT3* promoter drove posterior pole expression in only four out of nine transgenic lines (F), and neither *CUT2* (E) nor *TUC4* (H) conferred expression in endosperm (seven and 11 transgenic lines, respectively). The posterior pole of seeds 4 days after pollination was examined for expression of RFP (red). Auto-fluorescence detected on the GFP channel (green) highlights cellular structures in the endosperm and seed coat. Scale bars: 100 μm.

enrichment (Fig. 2D), that was sufficient to recapitulate the *AtFH5* expression pattern (Fig. 2E-H). In order to test whether this element was sufficient to confer imprinted expression, we used the *CUT4* transgenic reporter lines as male or female in crosses with wild-type plants (Fig. 3). H2B-mRFP was expressed maternally when *CUT4::H2B-mRFP* transgenic lines were pollinated with wild-type plants (Fig. 3A). When the same reporter lines were used as pollen donors, however, no RFP was detected in the endosperm (Fig. 3B). The expression of *CUT4::H2B-mRFP* thus recapitulates the imprinted expression of the endogenous *AtFH5* locus.

Loss of PcG activity in *mea* and *fis2* mutants caused paternal expression of *CUT4::H2B-mRFP* in the endosperm (Fig. 3C,D). Our results thus indicate that the *AtFH5* promoter alone is capable of directing FIS PcG-dependent imprinted expression of a reporter gene. We conclude that PcG-mediated silencing relies on sequence elements in the *AtFH5* promoter. In *Drosophila*, PcG activity is targeted by cis-acting Polycomb response elements (PREs) (Chan et al., 1994; Ringrose et al., 2003). The limited dissection of the *AtFH5* promoter suggests that it is likely to contain a PRE.

In addition, whereas *CUT4::H2B-mRFP* expression is confined to the posterior pole in the wild type (Fig. 2F and Fig. 3A), the reduction of PcG activity caused ectopic expression of maternally and paternally contributed *CUT4::H2B-mRFP* in the anterior and peripheral endosperm (Fig. 3C,D). The *MEA* and *FIS2* components of the PcG complex that is active in endosperm are expressed throughout endosperm until 2 days after fertilization, when their expression becomes confined to the posterior pole (Luo et al., 2000). Our results suggest that *AtFH5* expression is repressed by PcG activity in the anterior and peripheral endosperm. In the posterior, *MEA*, *FIS2* and *AtFH5* are co-expressed, suggesting that a repressor of PcG activity might be expressed at this location, resulting in *AtFH5* expression.

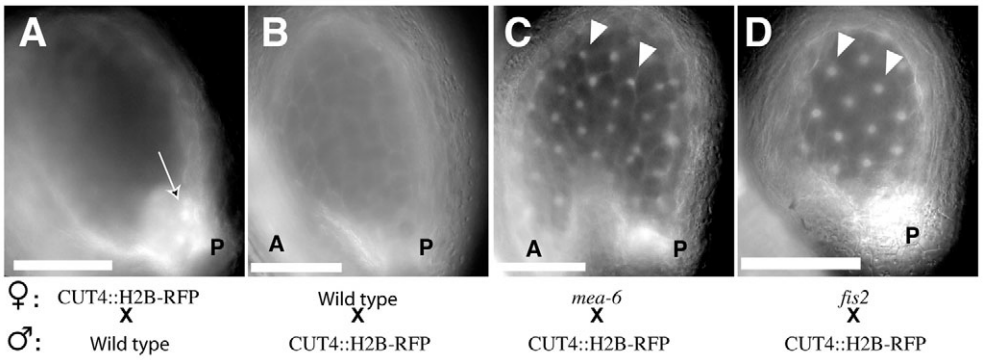

Fig. 3. Maternally patterned expression of the *CUT4::H2B-mRFP* reporter. (**A-D**) Seeds were examined for RFP expression 4 days after the indicated crosses. (A) Maternally derived *CUT4::H2B-mRFP* expression was limited to the posterior endosperm (arrow). However, paternal expression of *CUT4::H2B-mRFP* was not detected (B), unless the plant used as a mother in the cross was deficient for *MEDEA* (C) or *FIS2* (D). In these latter cases, red nuclei were detected throughout the peripheral and the anterior endosperm (arrowheads). Scale bars: 100 μm.

Morphogenetic consequences of *AtFH5* regulation by the PcG FIS complex

We studied the genetic interaction between *FIS* PcG regulation and *AtFH5* to uncover the role played by *AtFH5* in the complex endosperm phenotype caused by *FIS* gene maternal inheritance. We observed the endosperm phenotype in seeds produced by self-fertilization in *mea-6*/*mea-6*; *AtFH5*/*atfh5-1* plants. In comparison to the wild type (Fig. 4A), *mea*/*mea* seeds were larger, showed uncellularized endosperm, larger chalazal cysts and ectopic cysts in the peripheral endosperm (Fig. 4B) (n>500). In *atfh5*/*atfh5* seeds, the

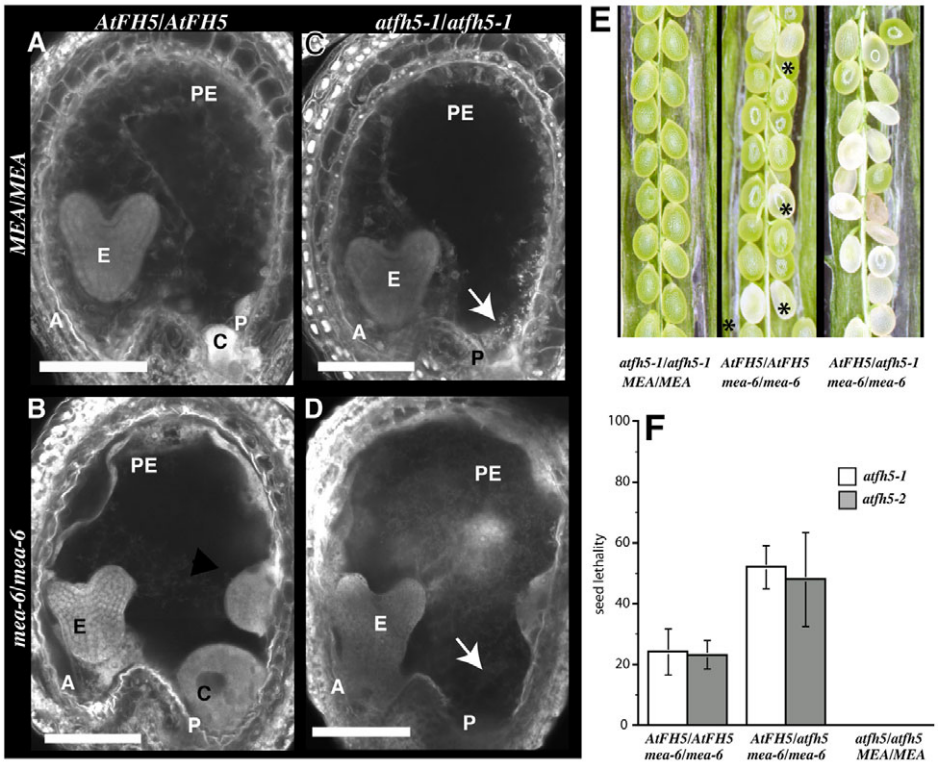

Fig. 4. Patterning defects in *mea-6 atfh5-1* double-mutant seeds. (**A-D**) Confocal microscopy of Feulgen-stained seeds from wild-type (A), *mea-6*/*mea*-6 (B), *atfh5-1*/*atfh5-1* (C) and *mea-6*/*mea-6*; *AtFH5*/*athf5-1* (D) plants. (A) Typical wild type showing cellularized endosperm and normal chalazal cyst. (B) A typical *medea* phenotype, which involves enlargement of the chalazal cyst and ectopic cyst structures (black arrowhead). (C) *atfh5-1* at the same stage has not fully cellularized and shows reduction of chalazal cyst (white arrow). (D) A seed from the *mea-6*/*mea-6*; *AtFH5*/*atfh5-1* background, which although homozygous for the *medea* mutation, is lacking the posterior cyst (white arrow). (**E**) Exposed seeds from siliques 7 days after pollination from a segregating F2 population of *mea-6* × *atfh5-1*. The indicated plant genotypes were established by PCR. White and collapsed seeds indicate the typical *fis* mutant lethality (selected examples are marked with asterisks). (**F**) Siliques as in A were scored for the percentage of white or collapsed seeds from F2 segregants of the *mea-6* × *atfh5-1* and *mea-6* × *atfh5-2* crosses. An increase of ~25% in lethality was observed between homozygous *mea-6* plants and *mea-6* plants heterozygous for either *atfh5-1* or *atfh5-2* [24.2±7.5% (n=397) to 52.1±7.1% (n=350) and 23.2±4.7% (n=336) to 48.0±15.5% (n=421), respectively]. Error bars represent s.d. of values obtained from at least three plants (n>100 seeds per plant). A, anterior pole; C, chalazal cyst; E, embryo; P, posterior pole; PE, peripheral endosperm. Scale bars: 100 μm.

posterior pole is missing, as described previously (Ingouff et al., 2005a) (Fig. 4C) (n>500). Since loss of FIS activity in this background results in the ectopic expression of *AtFH5* from either parental allele, we expected to observe a distinct double-mutant phenotype in those 25% of seeds that are homozygous for the *atfh5* mutation. We found that 26±3% of seeds produced by *mea-6*/*mea-6*; *AtFH5*/*atfh5-1* plants (n=303 seeds) and 23±4% of seeds produced by *mea-6*/*mea-6*; *AtFH5*/*atfh5-2* plants (n=319 seeds) showed an absence of cellularization and the overgrowth of endosperm typical of the *fis* mutant phenotype, but no large and ectopic cysts, which are also characteristic of the *fis* mutant phenotype (Fig. 4D) (n=303). The absence of posterior cyst in a *fis* mutant background correlated with a 25% increase in seed lethality in *mea-6*/*mea-6*; *AtFH5*/*atfh5-1* and *mea-6*/*mea-6*; *AtFH5*/*atfh5-2* plants (Fig. 4E,F). As a result, we could not obtain double-homozygous *mea*/*mea*; *atfh5*/*atfh5* plants. These observations support the hypothesis that the enlargement of the chalazal cyst observed in the *mea* endosperm specifically depends on the presence of *AtFH5*. *AtFH5* is required for the construction of the posterior endosperm and *mea* causes ectopic expression of *AtFH5* outside of the posterior endosperm. Presumably, ectopic actin organization by *AtFH5* delocalizes the recruitment of nuclei from the peripheral endosperm to the posterior pole and causes ectopic cyst formation in *fis* mutant endosperm. We thus conclude that *AtFH5* control of endosperm posterior pole morphogenesis is directly regulated by PcG.

Conclusions

We have identified *AtFH5* as a new imprinted gene in *Arabidopsis*. However, the imprinted status of *AtFH5* is not prefigured by a sex-specific pattern of expression in the gametes. This implies that specific activation of the maternal allele during female gametogenesis is not required for *AtFH5* imprinting, in contrast to other maternally expressed imprinted genes studied in *Arabidopsis*. The imprinted status is thus defined by silencing of the paternal allele followed by zygotic activation of the maternal copy. Similarly, most imprinted genes in mammals are not expressed during gametogenesis when the sex-dependent silencing occurs, and their imprinted status is revealed only later in specific tissues (Reik et al., 2001). *AtFH5* is silenced by PcG activity in vegetative tissues and in endosperm. It is likely that the repressive H3K27me2,3 marks are removed during female gametogenesis and that some transcriptional mechanism enables *AtFH5* expression only after fertilization in endosperm.

In animals and plants the genes marked by PcG complexes and associated with developmental regulation encode transcription factors that are important to establish patterning and cell fate (Ringrose and Paro, 2007; Schubert et al., 2005). Here, we show that PcG complexes may also directly control cytoskeletal genes required for proper morphogenesis. In support of this hypothesis, the *Drosophila* formin Diaphanous (Schwartz et al., 2006) and the mammalian diaphanous homologs (Bracken et al., 2006) were also identified as potential direct PcG targets in genome-wide profiling experiments. It is thus conceivable that a developmental mechanism has been conserved or selected independently during the evolution of multi-cellular eukaryotes, whereby PcG complexes regulate the transcription of genes that encode both transcription factors and structural molecules in order to create a patterned cellular context for physical processes involving cell migration and morphogenesis.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/20/3399/DC1

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