The *Polycomb*-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene *PHERES1*

Claudia Köhler,[1,4] Lars Hennig,[2] Charles Spillane,[1] Stephane Pien,[1,3] Wilhelm Gruissem,[2] and Ueli Grossniklaus[1]

[1]Institute of Plant Biology & Zürich-Basel Plant Science Center, University of Zürich, CH-8008 Zürich, Switzerland; [2]Institute of Plant Sciences, Swiss Federal Institute of Technology (ETH), ETH Zentrum, LFW E47, CH-8092 Zürich, Switzerland

The *Polycomb*-group (PcG) proteins MEDEA, FERTILIZATION INDEPENDENT ENDOSPERM, and FERTILIZATION INDEPENDENT SEED2 regulate seed development in *Arabidopsis* by controlling embryo and endosperm proliferation. All three of these FIS-class proteins are likely subunits of a multiprotein PcG complex, which epigenetically regulates downstream target genes that were previously unknown. Here we show that the MADS-box gene *PHERES1* (*PHE1*) is commonly deregulated in the *fis*-class mutants. PHE1 belongs to the evolutionarily ancient type I class of MADS-box proteins that have not yet been assigned any function in plants. Both MEDEA and FIE directly associate with the promoter region of *PHE1*, suggesting that *PHE1* expression is epigenetically regulated by PcG proteins. *PHE1* is expressed transiently after fertilization in both the embryo and the endosperm; however, it remains up-regulated in the *fis* mutants, consistent with the proposed function of the *FIS* genes as transcriptional repressors. Reduced expression levels of *PHE1* in *medea* mutant seeds can suppress *medea* seed abortion, indicating a key role of *PHE1* repression in seed development. *PHE1* expression in a hypomethylated *medea* mutant background resembles the wild-type expression pattern and is associated with rescue of the *medea* seed-abortion phenotype. In summary, our results demonstrate that seed abortion in the *medea* mutant is largely mediated by deregulated expression of the type I MADS-box gene *PHE1*.

[*Keywords: Arabidopsis*; *Polycomb* group; *MEDEA*; MADS-box; embryo; endosperm]

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The life cycle of higher plants alternates between a haploid gametophytic generation and a diploid sporophytic generation. The female and male gametes formed during the gametophytic phase fuse during fertilization to generate the sporophytic phase of the life cycle. Most sexually reproducing diploid plants undergo double fertilization, during which the egg cell and the homodiploid central cell are each fertilized by a sperm cell and give rise to the embryo and the triploid endosperm, respectively (Grossniklaus and Schneitz 1998).

In analogy to the Greek myth in which the priestess Medea killed her children Pheres and Meidos (Euripides 431 BC; Wolf 1996), a maternal effect mutant identified in a screen for gametophytic mutants in *Arabidopsis thaliana* was named *medea* (*mea*; Grossniklaus et al. 1998). Additional alleles of the *MEA* gene, termed *FERTILIZATION INDEPENDENT SEED DEVELOPMENT* (*FIS1*), as well as mutations at two other loci, *FIS2* and *FERTILISATION INDEPENDENT ENDOSPERM* (*FIE* or *FIS3*), were identified in genetic screens for mutants displaying seed development in the absence of fertilization (Ohad et al. 1996; Chaudhury et al. 1997). The three *fis*-class mutants show a gametophytic maternal effect: all seeds derived from a mutant female gametophyte (50% in a heterozygote) abort irrespective of the paternal allele. Early development of *fis* embryos is morphologically indistinguishable from that of wild-type siblings. However, *fis* embryogenesis is delayed after the globular stage and eventually arrests with oversized heart-shaped embryos surrounded by an abnormally proliferated en-

[3]Present address: Department of Plant Systems Biology, Gent University, Ledeganckstraat 35, B-9000 Gent, Belgium.
[4]Corresponding author.
E-MAIL ckoehler@botinst.unizh.ch; FAX 41-01-634-82-04.
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dosperm. Furthermore, the central cell of *fis* mutants undergoes division in the absence of fertilization, suggesting that the primary function of the *FIS* genes is to control cell proliferation (Grossniklaus et al. 2001).

The *MEA* gene encodes a *Polycomb*-group (PcG) protein for which the closest *Drosophila* homolog is Enhancer of Zeste [E(Z); Grossniklaus et al. 1998; Kiyosue et al. 1999]. Like E(Z), the MEA protein contains a characteristic SET domain, which was originally defined as a highly conserved domain found in the *Drosophila* Suppressor of variegation3–9, E(Z), and Trithorax proteins. The SET domain can confer histone methyltransferase activity (Yeates 2002). PcG proteins appear to maintain a transcriptionally repressed state of target genes during development by altering higher-order chromatin structure (Francis and Kingston 2001; Köhler and Grossniklaus 2002; Reyes et al. 2002).

E(Z) and the *Drosophila* PcG protein Extra sex combs (ESC) are part of a protein complex that also contains the PcG protein Suppressor of Zeste12 [SU(Z)12; Czermin et al. 2002; Müller et al. 2002]. FIE is homologous to ESC (Ohad et al. 1999) and has been shown to interact with MEA (Luo et al. 2000; Spillane et al. 2000; Yadegari et al. 2000). In addition, SU(Z)12 has homology with FIS2, and the similarity of the *fis2*, *mea*, and *fie* phenotypes as well as their overlapping expression patterns suggest that FIS2, FIE, and MEA likely function in a similar protein complex to regulate the expression of common target genes (Grossniklaus and Vielle-Calzada 1998; Luo et al. 1999, 2000; Spillane et al. 2000). PcG protein complexes in *Drosophila* and mammals regulate the expression of homeobox genes, and mutations in PcG genes cause homeotic transformations. Furthermore, PcG proteins regulate cell proliferation, and in humans their deregulated expression is associated with a range of cancers (Simon and Tamkun 2002). Interestingly, the only two PcG target genes that have been identified so far in plants encode MADS-box proteins, which are structurally unrelated to animal homeodomain proteins, but have similar homeotic functions in development. The CURLY LEAF (CLF) protein, which shares sequence similarity with MEA, regulates expression of the floral organ identity gene *AGAMOUS* (*AG*; Goodrich et al. 1997). Furthermore, VERNALIZATION2, which is similar to SU(Z)12, represses the expression of the *FLOWERING LOCUS C* (*FLC*) gene (Gendall et al. 2001).

Members of the MADS-box family of developmental genes have been identified in plants, animals, and fungi. Two types of MADS-box proteins (type I and type II) can be distinguished on the basis of their amino acid sequences in the MADS-box domain. Both AG and FLC are members of the type II MADS-box lineage, which had already diverged from the type I MADS-box lineage in a common ancestor of plants and animals (Alvarez-Buylla et al. 2000). To date, only type II MADS-box genes have been characterized at the functional level in plants (Ng and Yanofsky 2001).

Here we show that expression of the type I MADS-box gene *PHERES1* (*PHE1*) is tightly controlled by the FIS-class proteins MEA, FIE, and FIS2. We demonstrate that after fertilization *PHE1* is transiently expressed in the embryo and the endosperm of wild-type plants, whereas in *mea* and *fie* mutant seeds, *PHE1* remains highly expressed until the seeds abort. The FIS-class proteins are required for repression of *PHE1* after fertilization. A reduction of the elevated *PHE1* expression levels in *mea* mutant seeds rescues the *mea* seed-abortion phenotype, suggesting that *PHE1* plays a crucial function in the *MEA* regulatory pathway and is chiefly responsible for the lethal phenotype of *fis*-class mutants.

Results

Transcriptional profiling demonstrates that only a few genes are commonly deregulated in mea *and* fie *mutants*

To characterize changes in the transcriptional profile caused by loss-of-function mutations in genes encoding components of the MEA–FIE complex, we performed RNA profiling experiments using Affymetrix high-density oligonucleotide microarrays. Because we were particularly interested in early effects, we extracted RNA from siliques containing embryos at the preglobular stage (8–16-cell embryo). At this developmental stage, the morphology of *mea* and *fie* mutant seeds is indistinguishable from that of wild-type siblings, and the number of secondary transcriptional changes is therefore expected to be minimal. Furthermore, we performed our analysis with heterozygous mutants, as homozygous *mea/mea* mutant plants showed several phenotypic alterations that increased with inbreeding and most likely resulted from accumulation of epigenetic changes ("epimutations"; U. Grossniklaus, unpubl.). Siliques were harvested from mutant and wild-type control plants grown under the same conditions, and the stage of embryo development was confirmed by microscopic analysis of cleared seeds. In a first experiment, *mea/MEA* and Landsberg *erecta* (L*er*) wild-type plants were compared (RNA samples mea_1 and Ler_1); several weeks later *mea/MEA*, *fie/FIE*, and wild-type plants were compared in a second experiment (RNA samples mea_2, fie_2, and Ler_2). To minimize any effects of plant-to-plant transcriptional variation, material was harvested from at least 10 plants and pooled for each sample. After hybridization to Affymetrix Arabidopsis GeneChips, expression signals of 8247 different probe sets were analyzed. Signal intensities of 5217 probe sets (63%) were significantly higher than background (value of *P* in presence call of MAS5.0) on at least one chip. This set of filtered data was used for further analysis.

Because microarray data can contain high levels of noise, we constructed scatter plots of signal intensities for samples from mutants compared with intensities from the wild-type (Fig. 1A). In all three plots, the majority of data points cluster tightly along the diagonal, demonstrating the relatively low noise in individual experiments. Next, we calculated coefficients of variation (cv) as a quantitative measure of data quality. Comparison of samples derived from plants grown in parallel

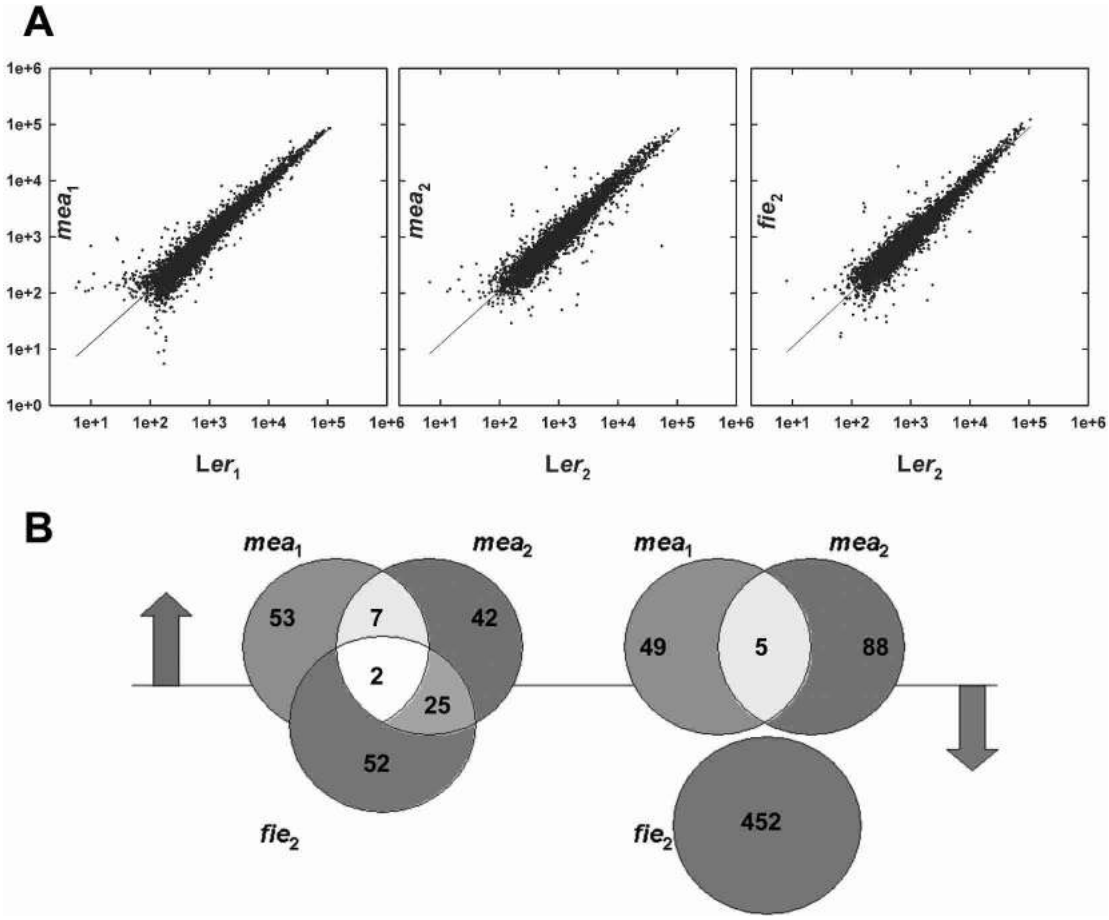

Figure 1. Transcriptional profiling of *mea* and *fie* mutants. RNA was isolated from siliques of *mea* (samples mea_1 and mea_2), *fie* (sample fie_2), and wild-type L*er* plants (samples Ler_1 and Ler_2). (*A*) Signal intensities of probe sets, which were flagged at least once present, were used to construct scatter plots. (*B*) Venn diagrams of potentially up-regulated (*left*) and down-regulated (*right*) genes. Note that transcripts of some genes are detected by more than one probe set; hence, the number of affected genes in Supplementary Table 1 is sometimes lower than the number of probe sets presented here.

(intraexperimental noise) yielded consistently low cv-values (14%–15%; Table 1). Interexperimental noise (comparisons between samples from plants grown independently) was substantially higher (24%). These results demonstrate that effects of organ sampling during harvest were considerably smaller than effects of unnoticed small differences in growth conditions between both experiments. Additionally, Table 1 includes the coefficient of variation for two RNA samples derived from wild-type seedlings grown 1 wk apart (WT_1 and WT_2). In this case the interexperimental noise (19%) was also higher than the intraexperimental noise of our samples from siliques, demonstrating the high quality of the data. Similarly, in a recent study analyzing the transcriptome of yeast grown in a chemostat, interexperimental noise of 20%–29% was reported (Piper et al. 2002).

Table 1. *Reproducibility of microarray data*

	mea_1 Ler_1	mea_2 Ler_2	fie_2 Ler_2	Ler_1 Ler_2	mea_1 mea_2	WT_1 WT_2
cv	14	15	14	24	24	19

Coefficients of variation are shown for different hybridizations as described in the text.

After comparative analysis of results from mutants with the corresponding wild type, 2749 probe sets (33%) were identified as having altered signal intensities in at least one of the three comparisons (values of *D* or *I* in change call of MAS5.0). We then selected probe sets that changed more than twofold and tested whether they were commonly affected in more than one RNA sample. According to our criteria, no probe set was down-regulated in all three mutant samples (Fig. 1B). In contrast, two probe sets in which we detected expression of a putative MADS-box transcription factor and a putative S-phase kinase-associated protein1 (Skp1), respectively, always exhibited increased transcript levels. Supplementary Table 1 lists descriptions and observed signal log ratios for all genes that were identified as affected in at least two of the three mutant samples. We observed that only the two aforementioned genes were affected consis-

tently in all three samples. In memory of the murdered sons of the mythological Medea, we named these candidate downstream targets of the *FIS*-class genes *PHERES1* (*PHE1*, At1g65330) and *MEIDOS* (*MEO*, At2g20160; Wolf 1996). In this study we focused our further analysis on the MADS-box gene *PHERES1* (*PHE1*).

PHE1 *is transiently expressed during seed development*

To characterize *PHE1* expression during stages of seed development other than those used for transcriptional profiling, we performed Northern blot analysis with RNA from defined developmental stages of wild-type, *mea/MEA*, and *fie/FIE* plants (Fig. 2A). We investigated *PHE1* expression in flowers before fertilization (Day 0), in seeds containing embryos at preglobular stages (1–2 d after pollination, DAP), and in seeds containing embryos at the late globular stage (3–4 DAP). Our analysis indicates that *PHE1* was not expressed to any significant level before fertilization in wild-type plants or in *mea/MEA* and *fie/FIE* mutants. Expression of *PHE1* increased strongly after fertilization in wild-type and mutant flowers. However, *PHE1* expression in both mutants was higher and remained at high levels until 4 DAP, whereas in wild-type flowers *PHE1* expression levels decreased again after 3 d. These results have been verified and extended in a different experiment that also included the *fis2/FIS2* mutant (Fig. 2B). Using reverse transcription followed by real-time PCR analysis, *PHE1* expression levels were quantified in wild-type and *fis*-class mutants at different stages of flower and seed development. In this experiment, we analyzed flowers before fertilization, open pollinated flowers (0–1 DAP), seeds containing embryos at the early globular stage (2–3 DAP), and seeds containing embryos at the late globular stage (3–4 DAP). Confirming our previous results, no *PHE1* expression is detectable before pollination. In wild-type flowers, *PHE1* expression became detectable in seeds containing preglobular-stage embryos and declined during later development. In contrast, *PHE1* expression in all three *fis*-class mutants initiated earlier than in wild-type plants, beginning directly after pollination. Furthermore, 2–3 DAP, *PHE1* mRNA levels in *fis*-class mutants exceeded wild-type *PHE1* expression levels by threefold (*mea/MEA*), 11-fold (*fie/FIE*), and ninefold (*fis2/FIS2*). Later during development, *PHE1* expression declined in wild-type seeds and also in the *fis* mutant seeds. However, in the *fis* mutant seeds, *PHE1* mRNA levels were consistently higher throughout development and above the maximum level observed for wild-type *PHE1* expression.

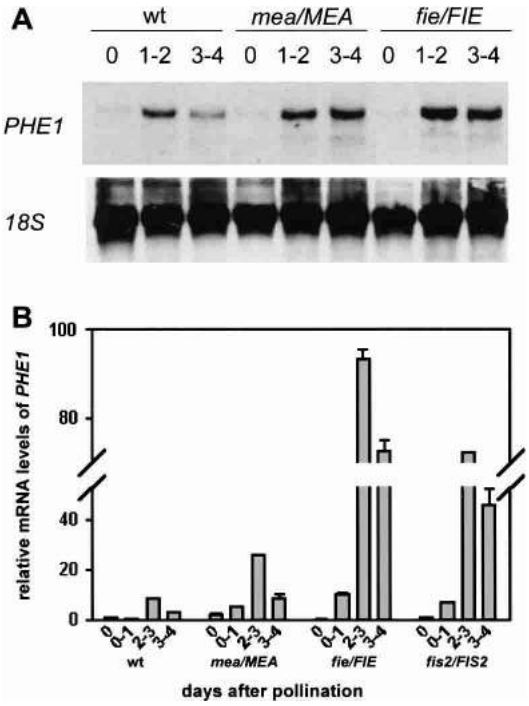

Figure 2. *PHE1* expression is transiently up-regulated in wild-type seeds and remains up-regulated in *fis* mutants. (*A*) Northern blot analysis of *PHE1* expression in wild-type and *mea/MEA* and *fie/FIE* mutants at different stages of seed development. RNA was isolated from flowers before fertilization (0 DAP), siliques containing embryos at the preglobular stage (1–2 DAP), and siliques containing embryos at late the globular stage (3–4 DAP). Equal loading of RNA was controlled by hybridization with an *18S* RNA probe. (*B*) Relative *PHE1* mRNA levels in wild-type seeds compared with *PHE1* expression in *mea/MEA*, *fie/FIE*, and *fis2/FIS2* mutants. RNA was isolated from flowers before fertilization (0 DAP), open pollinated flowers (0–1 DAP), siliques containing preglobular-stage embryos (1–2 DAP), and siliques containing late globular-stage embryos (3–4 DAP). mRNA levels of *PHE1* were quantitatively determined by real-time PCR and normalized with *Actin11* mRNA levels.

PHE1 *encodes a MADS-box transcription factor*

PHE1 encodes a MADS-domain protein (AGL37) of the evolutionarily ancient type I class (Alvarez-Buylla et al. 2000). The PHE1 protein comprises 279 amino acids and has a predicted molecular weight of 32 kD. The coding sequence of *PHE1* is not interrupted by introns. The amino acid sequence of PHE1 contains the highly conserved MADS-domain (59 amino acids) at the N terminus, followed by a region lacking any noticeable homology to other known protein domains. Within the MADS-domain, we observed a putative nuclear localization signal ($K_{22}RKK_{25}$), and transient expression studies in onion cells demonstrated that PHE1 is localized to the nucleus (data not shown).

Homology searches revealed the presence of a close homolog of *PHE1* (72% amino acid sequence identity), which we called *PHE2* (AGL38; At1g65300). *PHE2* is also located on Chromosome I ~10 kb distant to *PHE1*. We analyzed *PHE2* expression during seed development by real-time PCR and found that it is expressed in a similar pattern as *PHE1* in wild-type plants, but at eightfold lower expression levels compared with *PHE1* (data not shown). Similar to *PHE1*, *PHE2* expression in *fis* mutants is initiated earlier and rises to higher levels than in wild-type plants (data not shown).

PHE1 *expression overlaps with* MEA *expression*

MEA mRNA is detectable in the female gametophyte before fertilization and in the embryo and the endosperm after fertilization. In the embryo, *MEA* expression persists until the late heart and torpedo stage. In the endosperm, expression is mainly detected prior to cellularization, but persists in the chalazal region of the endosperm (Vielle-Calzada et al. 1999; Luo et al. 2000). To analyze where and when *PHE1* is expressed during seed development, we transformed *Arabidopsis* plants with a *PHE1*::*GUS* reporter gene cassette, consisting of 3.0 kb of 5′ *PHE1* promoter sequence ligated to the β-*glucuronidase* reporter gene (Jefferson 1987). Several independent *PHE1*::*GUS* transgenic lines displayed the same pattern of reporter gene expression. We observed similar expression patterns using a *PHE1*::*GUS* translational fusion construct (data not shown). Furthermore, we used in situ hybridization to determine the expression pattern of *PHE1* mRNA during seed development. As expected from the results obtained by Northern blot and real-time PCR, *PHE1* is not expressed in the female gametophyte before fertilization (Fig. 3A). *PHE1*::*GUS* expression as well as *PHE1* mRNA could first be detected 1–2 DAP in the preglobular-stage embryo and in the endosperm (Fig. 3B,M,N). In seeds containing globular-stage embryos, *PHE1* expression became restricted to the chalazal region of the endosperm before cellularization (Fig. 3C,O). Expression in this region of the endosperm could be detected until the late heart stage (Fig. 3D,E). Thus, *PHE1* expression overlaps with *MEA* expression after fertilization until embryos reach the globular stage and before the endosperm begins to cellularize. *PHE1*::*GUS* expression could not be detected in any vegetative or floral organs (data not shown).

PHE1 *expression is strongly up-regulated in developing* mea *and* fie *seeds*

To investigate the spatial and temporal deregulation of *PHE1* expression in developing *mea*/*MEA* and *fie*/*FIE* seeds, we analyzed *PHE1*::*GUS* expression in *mea*/*MEA* or *fie*/*FIE* mutant backgrounds. The *PHE1*::*GUS* expression pattern in *mea*/*MEA* or *fie*/*FIE* seeds containing preglobular embryos was indistinguishable from staining patterns we observed in wild-type *PHE1*::*GUS* seeds (data not shown). However, Figure 3F shows that when

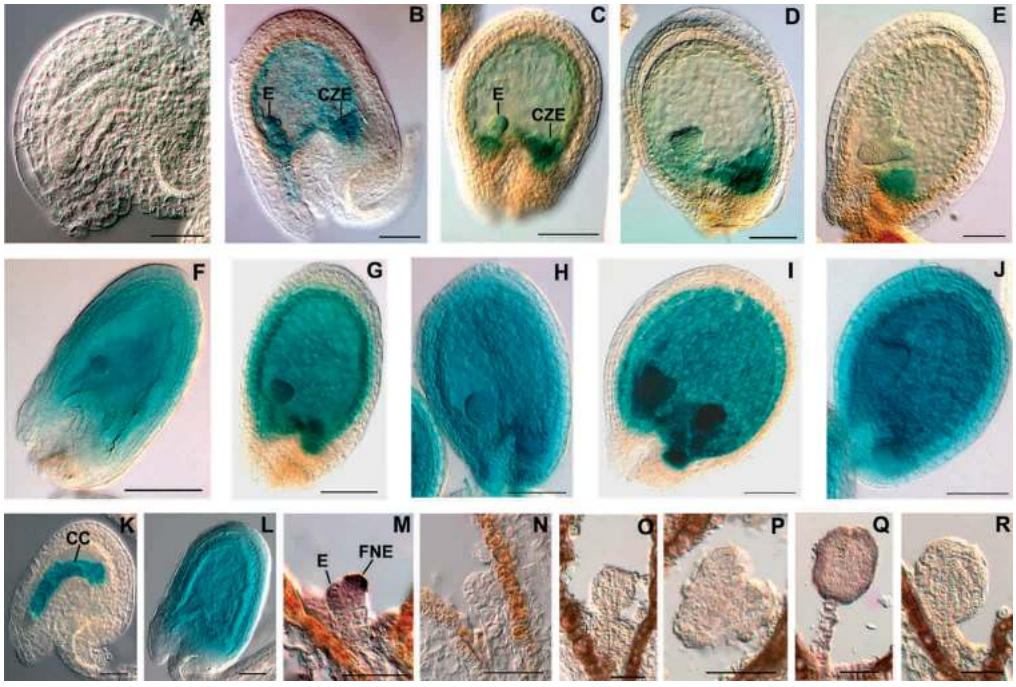

Figure 3. Localization of *PHE1* expression in gametophytes and developing seeds of wild-type *Arabidopsis* and *mea* and *fie* mutants. (*A–E*,*M–P*) Wild-type plants. (*F*,*G*,*I*,*Q*,*R*) *mea* mutants. (*H*,*J*,*K*,*L*) *fie* mutants. *A–L* are GUS stainings of plants transformed with a *PHE1*::*GUS* transgene. *M* and *O–Q* are hybridized with an antisense *PHE1* probe. *N* and *R* are hybridized with a sense *PHE1* probe. (*A*) *PHE1* is not transcribed in the female gametophyte prior to fertilization. (*B*) *PHE1* is expressed in the preglobular embryo (E). (*E*) *PHE1* is expressed in the endosperm with a stronger expression in the chalazal endosperm (CZE). (*C–E*) *PHE1* expression becomes restricted to the chalazal endosperm. (*F–J*) *PHE1* expression in *mea* and *fie* mutants remains detectable everywhere in the embryo and the endosperm. (*K*) *PHE1* expression starts in the central cell of *fie* mutant gametophytes 3 d after emasculation. (*L*) *PHE1* is expressed during autonomous endosperm development in *fie* mutants. (*M*) *PHE1* mRNA is localized in the preglobular embryo and in the free nuclear endosperm (FNE). (*N*) Preglobular embryo hybridized with a sense *PHE1* probe. (*O*) Globular embryo hybridized with an antisense *PHE1* probe. (*P*) Heart-stage embryo hybridized with an antisense *PHE1* probe. (*Q*) Overproliferated globular *mea* embryo hybridized with an antisense *PHE1* probe. (*R*) Overproliferated *mea* embryo hybridized with a sense *PHE1* probe. Bars: *A*,*K*,*M*,*N*, 30 μm; *B*,*L*,*O–R*, 50 μm; *C–J*, 100 μm.

embryos reached the globular stage, *GUS* expression was not restricted to the chalazal region of the endosperm but instead remained detectable in embryo and endosperm in ~50% of the seeds from plants homozygous for the *PHE1::GUS* transgene (*mea/MEA; PHE1::GUS/PHE1::GUS*: 186:202; blue seeds: colorless seeds, 1:1; $X^2 = 0.66 < \chi_{0.05[1]}^2 = 3.84$). *PHE1::GUS* expression in *mea/MEA* and *fie/FIE* seeds remained detectable at a very high level until embryos reached the heart stage and aborted (Fig. 3G–J). We confirmed the *GUS* expression patterns by in situ hybridization. *PHE1* mRNA could be detected in overproliferating globular *mea* embryos, whereas no *PHE1* expression was detectable in wild-type globular- or heart-stage embryos (Fig. 3O–R). Thus, MEA and FIE are necessary to down-regulate and restrict *PHE1* expression during seed development.

PHE1::GUS *is expressed in the absence of fertilization in* fie *gametophytes*

One characteristic phenotype of the *fis*-class mutants is their ability to initiate endosperm development in the absence of fertilization. The *FIS*-class genes are all expressed before fertilization, and most likely repress genes involved in endosperm development (Vielle-Calzada et al. 1999; Luo et al. 2000; Spillane et al. 2000). If the MEA–FIE complex represses *PHE1*, it is possible that in emasculated *mea* and *fie* mutants, *PHE1* expression could occur independently of a fertilization signal. To test this hypothesis, we emasculated *fie/FIE* and wild-type plants containing the *PHE1::GUS* transgene and analyzed *PHE1::GUS* expression. We focused our investigation on *fie/FIE* mutants, in which the fertilization-independent endosperm development phenotype is more highly penetrant than in *mea/MEA* mutant plants (Grossniklaus and Vielle-Calzada 1998). Over a period of 5 d, we analyzed six emasculated gynoecia per day from two wild-type and two *fie/FIE* mutant plants. In wild-type gametophytes, no *PHE1::GUS* expression could be detected up to 5 d after emasculation (data not shown). In contrast, several unfertilized gametophytes of *fie/FIE* plants showed strong *PHE1::GUS* expression, which was detectable 3 d after emasculation (Fig. 3K). *PHE1::GUS* expression began before the autonomous formation of endosperm nuclei and remained detectable when autonomous endosperm nuclei developed at 4 d after emasculation (Fig. 3L). Thus, repression of *PHE1* before fertilization is relieved in *fie/FIE* mutants and precedes autonomous endosperm formation.

The MEA–FIE Polycomb*-group complex is bound to the* PHE1 *locus*

The deregulated expression of *PHE1* in *mea* and *fie* mutants suggested that *PHE1* expression might be directly regulated by the MEA–FIE PcG complex. Hence, we investigated whether the MEA–FIE complex is directly bound to the *PHE1* locus and, if so, to which region(s) of the *PHE1* locus it may bind. Chromatin immunoprecipitation (ChIP) analysis was performed on nuclear extracts from *Arabidopsis* inflorescences containing closed flower buds, open flowers, and siliques containing embryos up to the late globular stage. We used affinity-purified antibodies (Abs) against MEA (α MEA Ab) or FIE (α FIE Ab). In nuclear extracts prepared from inflorescences, the α MEA Ab recognized a protein of ~100 kD, which was not recognized in nuclear extracts prepared from *mea/mea* mutant plants (Fig. 4A). The predicted molecular mass of the MEA protein is 79 kD, but it was previously reported to migrate in SDS polyacrylamide gels at ~100 kD (Yadegari et al. 2000). The α MEA Ab also detected a protein at ~90 kD in wild-type and *mea/mea* extracts that represents an unknown cross-reacting protein. The α FIE Ab detected a protein of ~40 kD (the calculated molecular mass is 41 kD) and an additional, slower-migrating protein in nuclear extracts from plants expressing a myc-tagged version of the FIE protein, demonstrating the specificity of the α FIE Ab (Fig. 4A). Chromatin from inflorescences was immunoprecipitated using the α MEA and α FIE Abs. The α MEA preimmune serum served as a negative control. Four regions of the *PHE1* locus were analyzed by PCR following the immunoprecipitations. These regions were located 660 bp upstream of the ATG start codon (fragment I), overlapping the ATG start codon (fragment II), at the end of the open reading frame (fragment III), and at the 3′-untranslated region of the gene (fragment IV; Fig. 4B). As a negative control, we analyzed the *ACTIN3* gene, which is not deregulated in *mea* and *fie* mutants (data not shown). Because the α MEA AB cross-reacted with an unknown protein, we performed immunoprecipitations using the α MEA and the α FIE ABs, which should give comparable results. ChIP using both Abs significantly enriched *PHE1* fragments I and II (compared with the preimmune serum), containing the *PHE1* upstream region and the beginning of the open reading frame (Fig. 4B,C). In contrast, no enrichment was found for *PHE1* fragments III and IV located at the 3′-region of the *PHE1* gene and in the 3′-untranslated region, respectively, or for the *ACTIN3* gene, demonstrating the specificity of the ChIP procedure. Similarly, enrichment relative to input instead of preimmune serum was three- to fourfold higher for *PHE1* fragments I and II than for *ACTIN3* (data not shown). These results show that the MEA–FIE complex is regulating *PHE1* expression by direct interaction with the *PHE1* promoter.

mea/mea; ddm1/ddm1 *double mutants show modified* PHE1 *expression*

Mutations in the chromatin-remodeling factor *DECREASE IN DNA METHYLATION1* (*DDM1*) can rescue the *mea* phenotype by activating the paternally inherited *MEA* wild-type allele if introduced from parents with a normally methylated genome (Vielle-Calzada et al. 1999). In contrast, in inbred homozygous *ddm1/ddm1* mutants that show global demethylation of the genome, no paternal wild-type *MEA* allele is required to rescue the seed-abortion *mea* phenotype (C. Spillane and U.

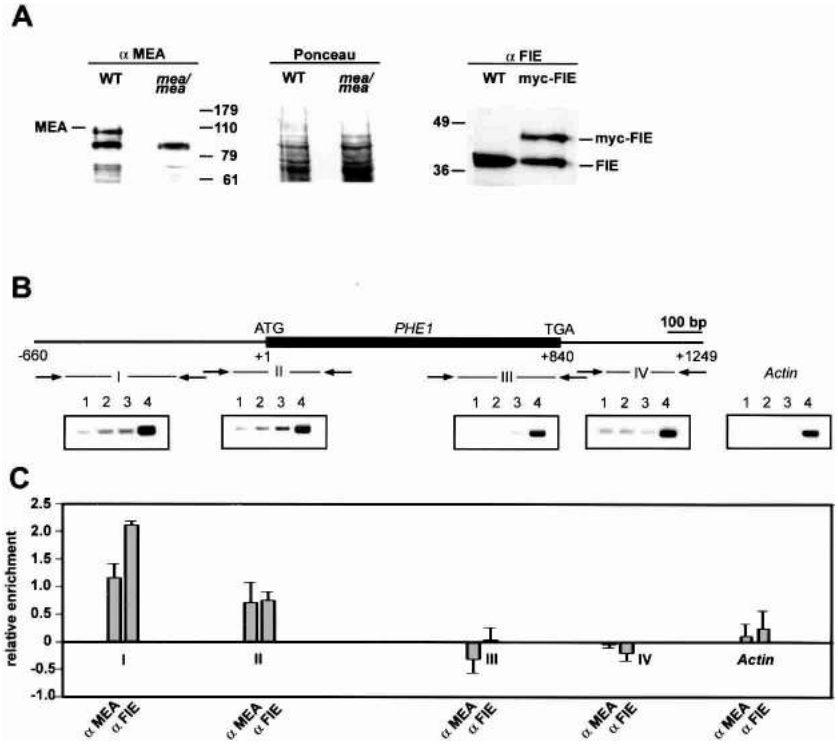

Figure 4. ChIP analysis to examine the presence of the MEA–FIE complex on the *PHE1* locus. (*A*) Characterization of the α MEA and α FIE Abs used. (*Left* panel) Nuclear extracts from wild-type or *mea/mea* plants were analyzed with the α MEA Ab. (*Middle* panel) Equal loading was controlled by staining the membrane with Ponceau. (*Right* panel) Nuclear extracts prepared from wild-type and plants containing a myc-tagged FIE version were analyzed with the α FIE Ab. (*B*) ChIP of the *PHE1* locus. Schematic diagram of the *PHE1* locus indicating the regions analyzed by PCR after ChIP. The numbers indicate the position relative to the translational start site of *PHE1*. Chromatin was prepared from wild-type inflorescences and immunoprecipitated using preimmune serum (PI, 1), α MEA Ab (2), and α FIE Ab (3). For input, 1% of the chromatin to be subsequently processed for ChIP was removed and PCR-amplified (4). The sizes of the PCR fragments are as follows: fragment I, 492 bp; fragment II, 410 bp; fragment III, 448 bp; fragment IV, 448 bp; *ACTIN3*, 490 bp. (*C*) Relative enrichment of PCR fragments after ChIP using α MEA and α FIE Abs compared with PI. PCR signals were quantified, and the relative enrichment (mean and standard deviation of three replicates) was plotted for each primer combination.

Grossniklaus, unpubl.). This bypass of *MEA* activity by global genome demethylation has also been demonstrated in antisense plants of the *DNA METHYLTRANSFERASE1* gene (Luo et al. 2000). These observations suggest that global changes in DNA methylation and chromatin structure may modulate downstream *MEA* targets by restoring their wild-type expression pattern in *mea* mutants. To test this hypothesis, we performed real-time PCR analysis of wild-type plants, homozygous *mea/mea* and *ddm1/ddm1* mutants, as well as *mea/mea; ddm1/ddm1* and *mea/mea; ddm1/DDM1* double mutants to test *PHE1* expression in these genetic backgrounds. The mutants analyzed were F4 individuals originally derived from F1 plants that were heterozygous for both mutations (i.e., *ddm1* and *mea*; Vielle-Calzada et al. 1999). Two homozygous *mea/mea* lines that were either homozygous (line A) or heterozygous (line B) for *ddm1* were initially identified in an F3 segregating population. The F4 progeny of these plants were used in this study. The demethylation effect of *ddm1* was confirmed by strongly reduced methylation at centromeric repeats in line A, whereas line B showed only weak changes of centromeric repeat methylation when assayed by Southern blot analysis using methylation-sensitive restriction endonucleases (Fig. 5A).

We investigated *PHE1* expression in lines A and B during different stages of seed development using flower buds before fertilization (Day 0), seeds containing embryos at preglobular stages (1–2 DAP), and seeds containing globular-stage embryos (3–4 DAP; Fig. 5B). *PHE1* expression in line A was significantly reduced compared with *PHE1* expression in *mea/mea* plants. This correlated with a dramatic rescue of seed abortion (63% normal seeds; $n = 487$) in comparison to homozygous *mea* mutants, which were wild-type for *DDM1* (0% normal seeds; $n = 485$). In contrast, *PHE1* expression in line B was similar to the expression pattern of *PHE1* in *mea/mea* mutants and remained high at later stages of embryo development. This was associated with only a small fraction of seeds that developed normally (6%; $n = 584$), which is not significantly different from that seen in hybrids between the genetic backgrounds of the *mea* and *ddm1* mutations (Vielle-Calzada et al. 1999). These results show that a reduced *PHE1* expression profile correlates with the *MEA*-independent rescue of seed abortion observed in these double mutants. We found no detectable expression of *PHE1* in other *ddm1/ddm1* mutant lines that were inbred for the same number of generations as line A. These results indicate a genetic interaction between the *mea* and *ddm1* mutations acting on *PHE1* expression.

The DDM1 protein belongs to the SWI/SNF family of chromatin-remodeling factors, and loss of DDM1 is known to cause both a global genome hypomethylation and a redistribution of methylation profiles at some loci (Kakutani et al. 1996; Jacobsen et al. 2000). Like DDM1, the *MORPHEUS MOLECULE* (*MOM*) gene also encodes a SWI/SNF-like protein that, similar to DDM1, can also release transcriptional gene silencing. However, in contrast to *ddm1* mutants in *mom* mutants, no changes in DNA methylation have been observed (Amedeo et al. 2000). To address the question of whether the *mom* mu-

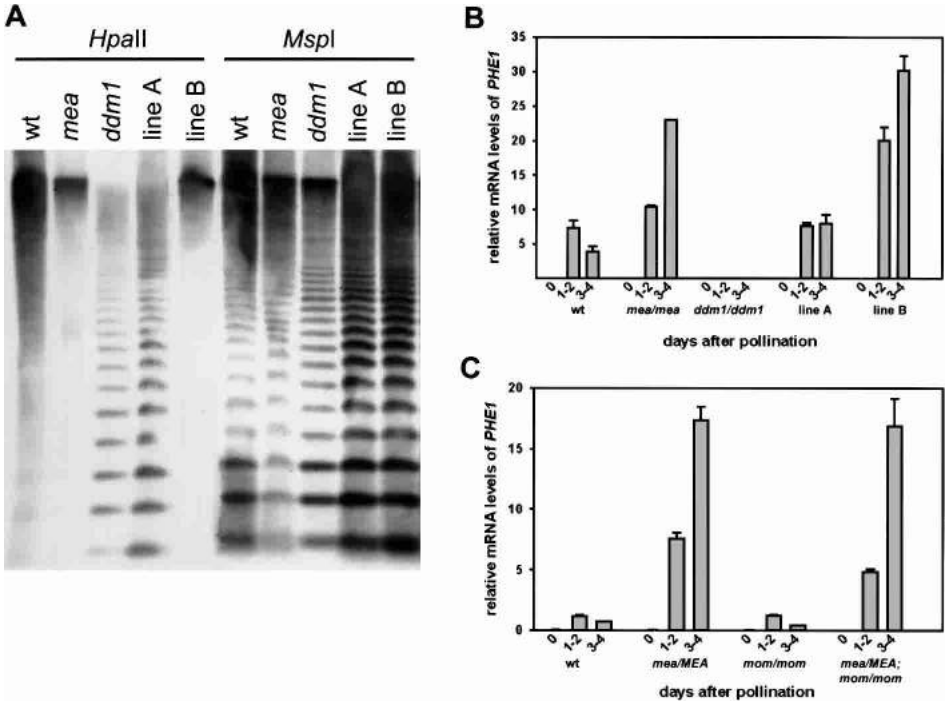

Figure 5. Analysis of DNA methylation and *PHE1* transcript levels in wild-type, *mea/mea*, *ddm1/ddm1*, *mom*, and double mutants. (*A*) Southern blot of genomic DNA of wild-type (wt), *mea/mea*, *ddm1/ddm1*, *mea/mea; ddm1/ddm1* (line A), and *mea/mea; ddm1/DDM1* (line B) double mutants restriction-digested with methylation-sensitive *Hpa*II (*left*) and methylation-insensitive *Msp*I (*right*), hybridized with the pericentromeric 180-bp repeat (Vongs et al. 1993). (*B*,*C*) Relative *PHE1* mRNA levels in wild-type (wt), *mea/mea*, *ddm1/ddm1*, and double-mutant lines A and B, as well as *mom/mom* and *mea/MEA; mom/mom* double mutants during different stages of seed development. RNA was isolated from flowers before fertilization (0 DAP), siliques containing embryos at the preglobular stage (1–2 DAP), and siliques containing embryos at the globular stage (3–4 DAP). mRNA levels of *PHE1* were quantitatively determined by real-time PCR and normalized with *Actin11* mRNA levels.

tation could also modify *PHE1* expression in an *mea* mutant background, we analyzed *PHE1* mRNA levels by real-time PCR in *mea/MEA; mom/mom* double-mutant plants (F3 generation) during different stages of seed development. We analyzed flower buds before fertilization (Day 0), seeds containing embryos at preglobular stages (1–2 DAP), and seeds containing globular-stage embryos (3–4 DAP; Fig. 5C). However, the *PHE1* expression profile of homozygous *mom* mutants and *mea/MEA; mom/mom* double mutants was indistinguishable from wild-type and *mea/MEA* plants, respectively. Furthermore, this was correlated with an unmodified seed abortion phenotype in *mea/MEA; mom/mom* mutants (53% normal seeds: 47% aborted seeds; $n = 773$; $X^2 = 3.24 < \chi_{0.05[1]}^2 = 3.84$).

The contrasting effects of the *ddm1* and *mom* mutants on both *PHE1* expression and *mea* seed abortion indicate a close connection between methylation changes, *PHE1* expression, and modification of the *mea* seed-abortion phenotype. In a hypomethylated *mea* mutant background, *PHE1* expression is down-regulated and the *mea* seed-abortion phenotype is rescued. In contrast, *PHE1* expression remains at high levels in *mea/mea; ddm1/DDM1* mutants and in *mea/MEA; mom/mom* mutants, which also show no changes in global methylation. In these latter cases, neither was any rescue of the *mea* seed-abortion phenotype observed.

Reduced PHE1 *expression can rescue* mea *seeds*

If high expression levels of *PHE1* in *mea* mutant seeds are causally linked with the *mea* seed-abortion phenotype and a wild-type expression profile of *PHE1* correlates with rescue from seed abortion as described above, a reduction of *PHE1* expression levels in a mutant *mea* background should shift *mea* seed-abortion ratios toward a higher proportion of normal seeds. We investigated this prediction by expressing the *PHE1* gene in antisense orientation (*asPHE1*) under control of the *MEA* promoter. The *MEApromoter::asPHE1* construct ensured that whenever *MEA* is expressed, *PHE1* levels should decrease because of the expression of antisense transcripts, which is expected to mimic the wild-type situation but in a mutant *mea* background. The expression characteristics of the chosen *MEA* promoter fragment was tested by analyzing *MEApromoter::GUS* plants. Using this reporter construct, *MEApromoter::GUS* expression was detected before fertilization in the gametophyte and after fertilization in the embryo and the endosperm until the late heart stage (data not shown). We obtained 21 independent transgenic *MEApromoter::asPHE1* lines in a *mea/MEA* mutant background and determined for each line the ratio of normal to aborting seeds. Normal seeds were defined by their phenotype as seeds having a wild-type shape and color. Seven of these transgenic lines

showed a significantly reduced number of aborting seeds, ranging from 38.5% to 46.4%, compared with the 50% aborting seeds consistently observed in untransformed *mea/MEA* plants (Fig. 6A). Interestingly, the seven *MEApromoter::asPHE1* lines not only showed a decrease in the frequency of aborting seeds but also formed seeds that were significantly larger than their wild-type siblings (1.6× larger in volume than wild-type; $n = 21$ enlarged seeds; $n = 58$ wild-type seeds). When wild-type seeds were completely developed and turned brown, the enlarged seeds were still green, indicating a developmental delay (Fig. 6B). Enlarged seeds were observed at a frequency of 1.9%–5.6% in different transgenic lines. The shift of aborting seeds toward normal or enlarged seeds was transmitted to the next generation of transgenic plants (Fig. 6A). As the occurrence of enlarged seeds was dependent on the presence of the *MEApromoter::asPHE1* transgene, they most likely represent mutant *mea* seeds that were rescued by reduced expression of the downstream target gene *PHE1*. Therefore, enlarged seeds could be either heterozygous (*mea* maternal allele) or homozygous for the *mea* mutation. We tested this hypothesis by genotyping plants derived from such enlarged seeds. Among 12 plants obtained from four different lines, we found that six of them were heterozygous for the *mea* mutation and six were homozygous (Fig. 6C). Thus, the presence of the *MEApromoter::asPHE1* transgene can rescue the *mea* phenotype independently of a wild-type *MEA* allele, suggesting that *PHE1* is chiefly responsible for the *mea* seed-abortion phenotype.

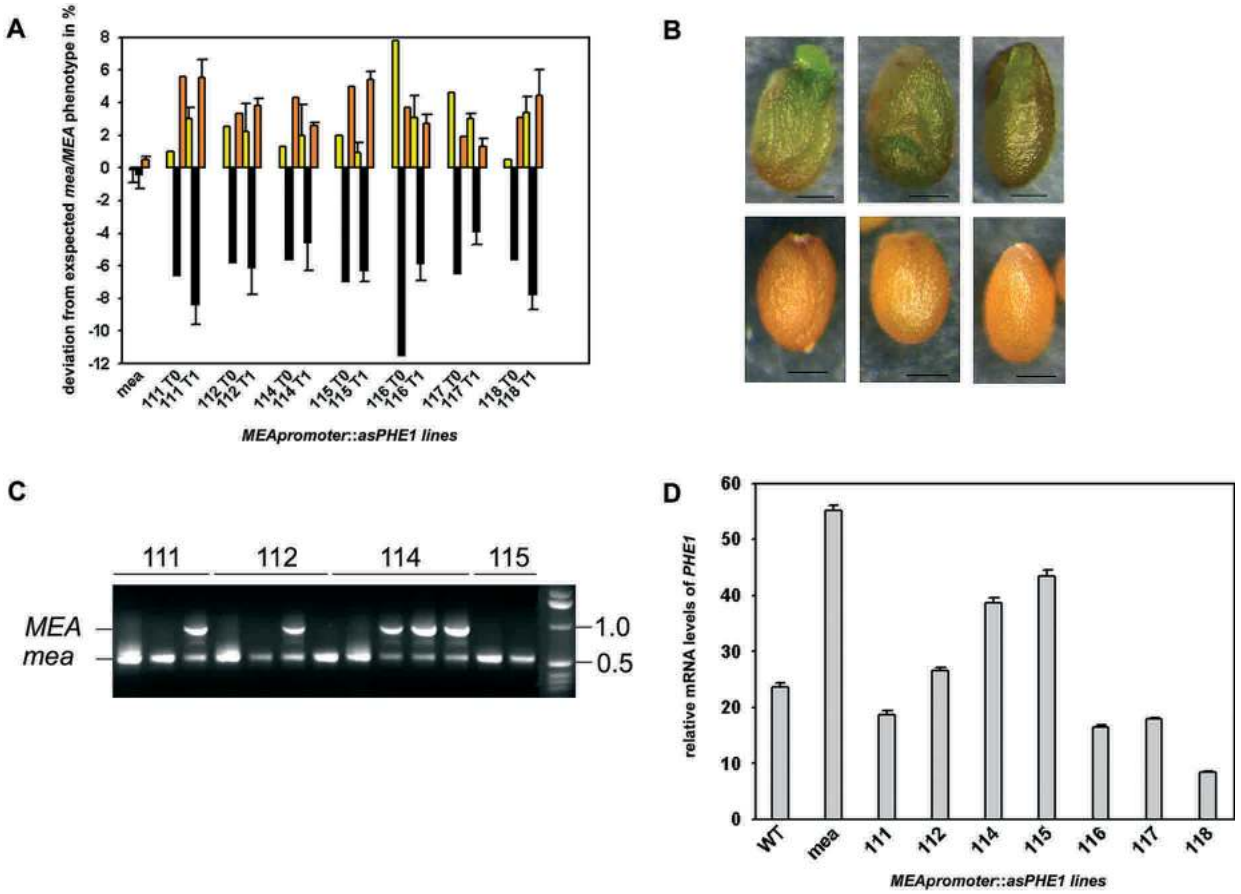

Figure 6. Reduced *PHE1* expression can rescue *mea* seeds. (*A*) Seed-abortion ratios were determined from seven *mea/MEA; MEApromoter::asPHE1* lines. Seed-abortion ratios are indicated as the deviation from the expected ratio of 50% normal to 50% aborted seeds. Normal seeds are represented by yellow bars, aborted seeds by black bars, and enlarged seeds by orange bars. From each line, this ratio was determined for the T0 and the T1 generation. For the numbers of the T1 generation, three different plants were counted. Numbers of counted seeds are as follows: line 111, T0: $n = 514$, T1: $n = 1243$; line 112, T0: $n = 516$, T1: $n = 1418$; line 114, T0: $n = 671$, T1: $n = 1066$; line 115, T0: $n = 465$, T1: $n = 1206$; line 116, T0: $n = 187$, T1: $n = 1056$; line 117, T0: $n = 269$, T1: $n = 1364$; line 118, T0: $n = 422$, T1: $n = 1171$. Numbers for *mea/MEA* plants were obtained from three different plants grown under same conditions as the *mea/MEA; MEApromoter::asPHE1* T1 plants ($n = 1450$). (*B*) Enlarged seeds of three different transgenic lines (111, 115, 118 from *left* to *right*; *top*) are significantly larger than their wild-type siblings (*bottom*). Bars, 300 µm. (*C*) PCR-based genotyping of plants originated from enlarged seeds. The *lower* bands correspond to *mea* mutant alleles, the *upper* bands correspond to *MEA* wild-type alleles. (*D*) *PHE1* mRNA levels in seeds of different *mea/MEA; MEApromoter::asPHE1* lines compared with *PHE1* expression levels in wild-type and *mea/MEA* seeds. Expression of *PHE1* in seeds containing late globular-stage embryos was quantitatively determined by real-time PCR and normalized with *Actin11* mRNA levels.

The expression level of *PHE1* was analyzed in the seven selected transgenic *MEApromoter::asPHE1* lines by quantitative PCR using RNA isolated from siliques containing late globular-stage embryos (3–4 DAP; Fig. 6D). All lines showed reduced *PHE1* mRNA levels (15.3%–78.9%) in comparison to *PHE1* expression observed in *mea/MEA* mutants. These results demonstrate that reduced expression of the downstream target gene *PHE1* in *mea* mutant seeds can suppress *mea* seed abortion. However, we did not observe a linear correlation between the level of *PHE1* reduction and the ratio of normal or enlarged to aborting seeds. It is possible that *PHE1* expression is essential in a narrow time window during seed development, and that such an expression status can only be recapitulated in some antisense lines by reducing *PHE1* expression in *mea* mutant seeds. There was no indication that reduced *PHE1* expression had any detrimental effect on wild-type seed development. Expression of the *MEApromoter::asPHE1* construct in a *MEA* wild-type background did not cause any seed-abortion effects (data not shown).

Enlarged mea *seeds rescued by the* MEApromoter::asPHE1 *transgene show abnormal development*

At maturity, the viability of the enlarged seeds was not significantly reduced compared with wild-type seeds. However, after complete desiccation and storage for several months, germination efficiencies were lower for enlarged seeds (74.4%; $n = 98$) than for wild-type seeds (98%; $n = 100$). Aberrant embryo and endosperm development within the enlarged seeds was observed for different transgenic lines. We observed embryos with almost normal morphology and bent cotyledons (22.5%; $n = 40$). However, the diameter of the cotyledons and the hypocotyl was consistently larger than in wild-type embryos (Fig. 7A–C). Furthermore, a large proportion of cellularized endosperm remained between the cotyledons and hypocotyl (Fig. 7F). Cotyledons derived from such embryos contained smaller, undifferentiated cells interspersed among normally differentiated mesophyll cells (Fig. 7G,H). In the enlarged hypocotyls, the cells were ~25% wider than cells in wild-type embryos (cells of the second cell layer below the epidermis were measured) in a region from 80 µm to 200 µm from the root tip (wild-type: $n = 47$; enlarged embryos: $n = 50$; Fig. 7I,J). The majority of enlarged seeds contained embryos (67.5%; $n = 40$) with an enlarged hypocotyl but smaller cotyledons compared with wild-type embryos (Fig. 7D). The space that was not occupied by the cotyledons was filled with cellularized endosperm. A small fraction of embryos (10%; $n = 40$) had an abnormal morphology with unbent cotyledons (Fig. 7E). The cotyledons were perpendicular to the extremely enlarged hypocotyl. The whole embryo was surrounded by large amounts of endosperm. Seeds containing embryos of this type had a round shape and appeared swollen.

Thus, in contrast to *mea* mutant embryos that typically arrest their development upon reaching heart stage, *mea* mutant embryos with reduced *PHE1* expression can proceed further in development, and many are fully viable. However, the developmental defects and the reduced germination efficiency after desiccation indicate that decreased *PHE1* expression alone is not sufficient to completely restore normal seed development.

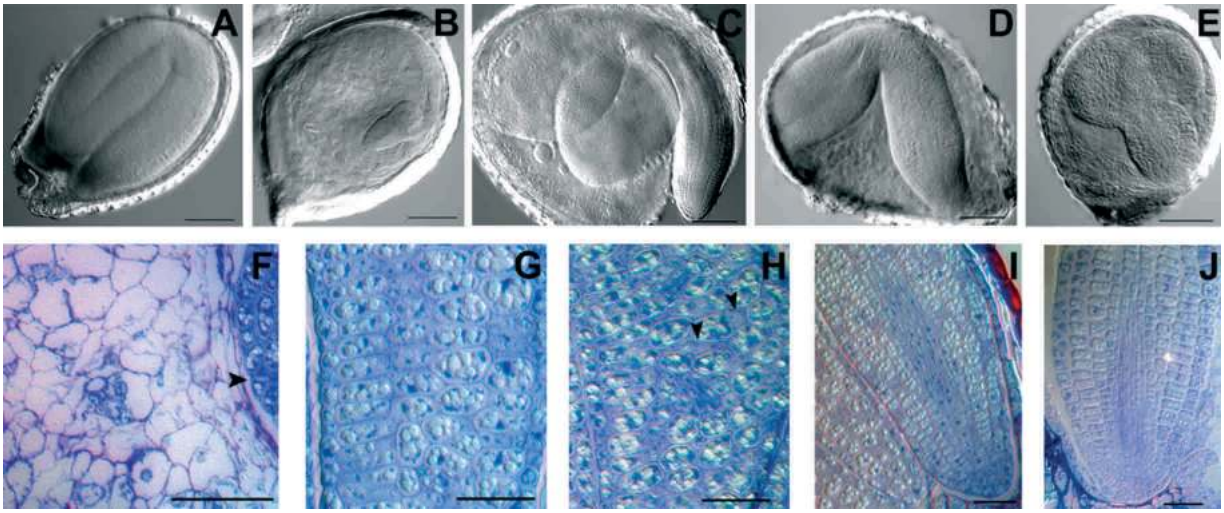

Figure 7. Morphology of embryos of enlarged seeds. (*A*) Normally developed wild-type embryo. (*B*) *mea* embryo shortly before abortion. (*C–E*) *mea/MEA; MEApromoter::asPHE1* embryos with different degrees of phenotypic alterations. (*C*) Overproliferated embryo with developed cotyledons. (*D*) Embryo with reduced cotyledon size and enlarged hypocotyl. (*E*) Embryo with cotyledons perpendicular to the swollen hypocotyl. (*F*) Section of cellularized endosperm within *mea/MEA; MEApromoter::asPHE1* seeds. The position of the embryo is indicated by an arrowhead. (*G*) Sagittal section of a wild-type cotyledon. (*H*) Sagittal section of an enlarged cotyledon of a *mea/MEA; MEApromoter::asPHE1* embryo. Small undifferentiated cells are indicated by arrowheads. (*I*) Longitudinal section through hypocotyl of wild-type seedling. (*J*) Longitudinal section through enlarged hypocotyl of *mea/MEA; MEApromoter::asPHE1* seedling. Bars: *A–E*, 100 µm; *F–J*, 20 µm.

Discussion

A type I MADS-box gene is an early downstream target of the MEA–FIE complex

To identify downstream target genes of the FIS-class PcG genes *MEA* and *FIE*, we conducted an expression profiling screen using Affymetrix GeneChip technology. When the expression profiles of the mutants *mea*, *fie*, and the wild-type Landsberg *erecta* were compared during early embryogenesis, we discovered only two genes that were significantly deregulated in both of the mutants. The identified genes were up-regulated before the onset of observable phenotypic alterations within mutant seeds, suggesting that they are early target genes of the MEA–FIE complex. The absence of any gene with lower expression levels in both mutants supports the view that the MEA–FIE complex functions as a transcriptional repressor. Our results indicate that deregulated expression of very few target genes in *fis*-class mutants appears to be sufficient to initiate aberrant development leading to seed abortion. One of the two target genes we detected codes for a Skp1-like protein. Such proteins are core components of the SCF ubiquitin ligase complex that marks cell cycle regulators and transcription factors for degradation (Tyers and Jorgensen 2000). This gene is therefore an interesting candidate target gene of the MEA–FIE complex, which could control cell proliferation and differentiation. Further expression analysis indicated that this gene is indeed up-regulated in *mea* and *fie* mutants (data not shown).

In this study, we have focused our analysis on the second target gene, encoding the MADS-box protein PHE1. The MADS-domain is a conserved sequence motif involved in DNA binding and dimerization (Pellegrini et al. 1995; Sharrocks and Shore 1995). MADS-domain proteins have been found in all eukaryotic kingdoms. Phylogenetic analysis of the MADS-domain suggests that an ancestral MADS-box gene duplication had occurred before the divergence of plants and animals ~2500 million years ago (Mya), forming two distinct clades of MADS-box proteins (type I and II lineages), which are present in both the plant and animal kingdoms (Alvarez-Buylla et al. 2000). All plant MADS-box proteins that have been functionally characterized so far belong to the type II lineage. Type II MADS-box proteins are involved in many critical aspects of plant development such as regulation of floral organ and meristem identity, induction of flowering, as well as development of ovules, fruits, leaves, and roots (Ng and Yanofsky 2001). The type I MADS-box lineage in plants is more closely related to the previously identified animal SRF-like MADS-box genes (Alvarez-Buylla et al. 2000). Genes in this class have not yet been assigned any function in plants. It is therefore interesting that the target gene *PHE1* belongs to the type I class of MADS-box proteins, which contain no known structural domains other than the conserved MADS-box. Our results provide the first insights into the function of a type I MADS-box gene in plants and furthermore demonstrate that *PHE1* is a key downstream target of the MEA–FIE complex, which plays a critical role in seed development.

Two other MADS-box genes (*AG* and *FLC*) of the evolutionarily distant type II lineage have previously been described as potential target genes of plant PcG proteins (Goodrich et 1997; Gendall et al. 2001). Even though the PcG protein complexes may be similar, the regulatory targets of PcG proteins in plants contrast with those in animals, where PcG proteins mainly act as transcriptional repressors of homeobox gene targets, which are structurally unrelated to MADS-box genes. However, both MADS-box proteins in plants and homeodomain proteins in animals have been recruited for the regulation of analogous developmental processes, for example, the specification of organ identity. Therefore, the expression of these developmental master switches is tightly controlled by evolutionarily conserved mechanisms (Köhler and Grossniklaus 2002).

PHE1 *expression is up-regulated in* mea, fie, *and* fis2 *mutants*

PcG proteins are proposed to maintain genes in a transcriptionally repressed state, which has been initially established by other proteins (Simon and Tamkun 2002). This is also the case for the repression of *PHE1* by the FIS-class proteins prior to fertilization. *PHE1* remains repressed in the female gametophytes of *fis* mutants, and derepression of *PHE1* in *fis* mutants is not immediate and only begins after 3 d if fertilization did not occur. After fertilization, *PHE1* expression begins earlier and reaches higher expression levels in *fis* mutant seeds compared with wild-type seeds. *PHE1* expression within the embryo and endosperm overlap both temporally and spatially with *MEA* and *FIE* expression (Vielle-Calzada et al. 1999; Luo et al. 2000; Spillane et al. 2000). Therefore, it is likely that the repressive effect of MEA and FIE on *PHE1* transcription requires other factors, such as additional components of the MEA–FIE complex or independently acting transcription factors. Because the general pattern of transient *PHE1* expression is still recognizable in *fis* mutants, it is likely that the FIS-class proteins (similar to other PcG proteins) also act to maintain transcriptional states established by other inductive and repressive factors. Our data indicate that MEA and FIE maintain a restricted *PHE1* expression pattern in certain regions of the embryo and endosperm after fertilization. Whereas *PHE1* expression in wild-type seeds becomes restricted to the chalazal region of the endosperm, it remains expressed uniformly within the embryo and the endosperm in *mea* and *fie* mutants. The deregulated *PHE1* expression within the endosperm could contribute to the observed endosperm defects of *fis* mutants. The *fis* mutants show a perturbed endosperm anterior–posterior axis with an enlargement of the chalazal region relative to other parts of the endosperm (Sorensen et al. 2001). As MADS-box proteins can regulate pattern formation during the establishment of organ identity, it is tempting to speculate that *PHE1* is involved in pattern formation of the endosperm. Furthermore, deregulated *PHE1* expres-

sion in *fis*-class embryos could lead to a progressive cell proliferation uncoupled from cell differentiation, giving rise to overproliferated heart-stage embryos. Up-regulation of *PHE1* expression is significantly stronger in *fie/FIE* and *fis2/FIS2* mutants compared with the *mea/MEA* mutant. It will be interesting to determine whether weaker expression of *PHE1* in seeds of *mea/MEA* mutants compared with *fie/FIE* and *fis2/FIS2* mutants is caused by a partial functional redundancy of proteins, such as EZA1 and CLF, which are similar to MEA (Baumbusch et al. 2001).

Our studies show that *PHE1* expression is commonly up-regulated in *mea*, *fie*, and *fis2* mutants, even though the extent of up-regulation can differ among the *fis* mutants. These findings support the hypothesis that the FIS proteins are part of a common protein complex regulating common target genes (Grossniklaus and Vielle-Calzada 1998; Luo et al. 2000; Spillane et al. 2000; Yadegari et al. 2000).

MEA and FIE are associated with the PHE1 *locus*

In *Drosophila*, PcG complexes exert their epigenetic regulatory function by binding to downstream Polycomb Response Elements (PREs) and to core promoters. The PRE is a region consisting of several elements that act together to maintain epigenetically silenced transcription patterns (Francis and Kingston 2001). Interestingly, in mammals no PRE has yet been identified. Using ChIP, we show that the plant PcG proteins MEA and FIE are directly associated with the *PHE1* locus. This demonstrates that *PHE1* is, indeed, a primary target gene of the MEA–FIE PcG complex and, thus, the first unequivocally identified primary target of a PcG complex in plants. Significantly, we found strong association of MEA and FIE with the 5′ end of the *PHE1* locus overlapping the putative transcriptional start (41 bp upstream of the ATG; data not shown). This suggests that the MEA–FIE complex contacts the basal transcription machinery, as it has been reported for other PcG complexes in animals (Orlando 2003).

Global methylation changes modify PHE1 *expression levels and rescue the* mea *seed-abortion phenotype*

Our data suggest that the suppressive effect of inbred *ddm1* mutations on the *mea* seed-abortion phenotype can result from a restoration of the wild-type-like expression patterns of downstream target genes of MEA. The DDM1 protein has homology to the SWI/SNF family of chromatin-remodeling factors, and may have an indirect role in modulating DNA methylation patterns (Jeddeloh et al. 1999). DDM1 appears to be required for the maintenance of histone H3 methylation patterns at heterochromatic sites, suggesting that in *ddm1* mutants, H3K9 methylated histones are redistributed from heterochromatic to euchromatic regions, giving rise to an activation of transcriptionally silenced loci (Gendrel et al. 2002). In contrast to DDM1, the SWI/SNF-like protein MOM is required for the maintenance of transcriptional gene silencing independent of changes in methylation across the silenced locus. MOM may function either downstream of methylation or in a pathway acting independently of methylation (Amedeo et al. 2000). The observation that a reduced *PHE1* expression within an *mea* mutant background was only observed in a hypomethylated *ddm1* mutant background suggests that global changes in chromatin structure can change the expression of *PHE1* by either changing its DNA and/or histone methylation status, or by altering its subnuclear localization. Initial studies indicate that there are, indeed, differences in the DNA methylation profile among wild-type plants, *mea* mutants, and *ddm1* mutants (C. Köhler, unpubl.). However, the unambiguous identification of those DNA or histone methylation marks set by the MEA–FIE complex will ideally require the isolation of tissue where MEA and FIE are expressed and will be subject to future investigations.

Enlarged mea/mea; MEApromoter::asPHE1 *seeds resemble* mea/MEA; ddm1/ddm1 *seeds*

We observed striking similarities between enlarged seeds with the genotype *mea/mea; MEApromoter::asPHE1* and the enlarged seeds with the genotype *mea/MEA; ddm1/ddm1* previously described (Vielle-Calzada et al. 1999). Similar to the *mea/MEA; ddm1/ddm1* enlarged seeds, we observed embryos with comparable developmental defects that are reminiscent of the *mea* phenotype, a developmental delay and overproliferated embryo and endosperm, but are viable. In a segregating seed population where *ddm1* was introduced from normally methylated parental genomes, all enlarged seeds had the genotype *mea/MEA; ddm1/ddm1*, suggesting that delayed activation of the paternally suppressed imprinted *MEA* allele confers the enlarged seed phenotype (Vielle-Calzada et al. 1999). Here *mea* seeds are rescued via another route leading to enlarged seeds, with the rescuing effect of the *MEApromoter::asPHE1* transgene being independent of a wild-type paternal *MEA* allele. This fully supports our hypothesis that *PHE1* acts downstream of *MEA*, and indicates that upstream requirements for *MEA* activity can be partially replaced by restoring a nearly wild-type expression pattern of the downstream target gene *PHE1*. The phenotypic alterations of the enlarged *mea* seeds carrying the rescuing transgene could be either a consequence of remaining deregulation of further potential *MEA* target genes, a delay in down-regulation of *PHE1* using the antisense approach, or result from an only partially restored *PHE1* expression pattern.

In conclusion, PcG proteins in both plants and animals have been structurally and functionally conserved during evolution as repressors of homeotic genes. Our study demonstrates that *PHE1* is a key downstream target that is transcriptionally repressed by the multiprotein PcG complex containing the FIS-class gene products, MEA and FIE. Interestingly, PHE1 belongs to the type I class of MADS-box proteins to which no functions have been assigned so far. This report provides the first indication

that both type I and type II MADS-box genes can be subject to repression mediated by evolutionarily conserved PcG multiprotein complexes. The epigenetically regulated target gene *PHE1* plays an important role during seed development, as overexpression of *PHE1* is largely responsible for the *mea* seed-abortion phenotype. Overall, our transcript profiling approach has facilitated the isolation of the first primary target gene of the MEA–FIE PcG complex that plays a crucial role during seed development, and provides the first demonstration of a fundamental role for a type I MADS-box protein in plants.

Materials and methods

Plant material and growth conditions

The *mea* mutant used in this study was the *mea-1* allele described by Grossniklaus et al. (1998). The *fie-2* mutant was kindly provided by Abed Chaudhury (CSIRO Division of Plant Industry, Canberra, Australia; Chaudhury et al. 1997). The *fis2-2* mutant was isolated in our lab (D. Page, unpubl.). The *ddm1-2* mutant was kindly provided by Eric Richards (Department of Biology, Washington University, St. Louis, MO; Vongs et al. 1993). For details on plant growth conditions, see the Supplemental Material.

High-density oligonucleotide array expression analysis

Preparation of cDNA and biotin-labeled cRNA were performed as recommended by Affymetrix. For details, see the Supplemental Material.

Northern and Southern blot analysis and real-time PCR

Genomic DNA was prepared with Nucleon Phytopure (Amersham Pharmacia Life Science), and RNA was prepared with TRIzol reagent (GIBCO-BRL) according to the suppliers' recommendations. Procedures for Northern and Southern blot analysis have been described (Ausubel et al. 1995). Real-time PCR procedures are described in the Supplemental Material.

Generation of transgenic plants

Detailed descriptions of plasmids and procedures for the generation of transgenic plants are described in the Supplemental Material.

Genotyping mea/MEA *plants by PCR*

For detailed descriptions of the PCR conditions, see the Supplemental Material.

In situ hybridization

In situ hybridization was performed as described (Vielle-Calzada et al. 1999) with modifications described in the Supplemental Material.

Histological analysis

Whole-mount clearings were performed as described (Vielle-Calzada et al. 1999). For GUS expression analysis, see the Supplemental Material.

Chromatin immunoprecipitation

The ChIP protocol used in this study is a modified version of a previously published protocol (Johnson et al. 2002). For details, see the Supplemental Material.

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Claudia Köhler, Lars Hennig, Charles Spillane, et al.

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