Dynamic regulatory interactions of *Polycomb* group genes: *MEDEA* autoregulation is required for imprinted gene expression in *Arabidopsis*

Célia Baroux,¹ Valeria Gagliardini,¹ Damian R. Page,² and Ueli Grossniklaus³

Institute of Plant Biology and Zürich-Basel Plant Science Center, University of Zürich, CH-8008 Zürich, Switzerland

The imprinted *Arabidopsis Polycomb* group (PcG) gene *MEDEA* (*MEA*), which is homologous to *Enhancer of Zeste* [*E(Z)*], is maternally required for normal seed development. Here we show that, unlike known mammalian imprinted genes, *MEA* regulates its own imprinted expression: It down-regulates the maternal allele around fertilization and maintains the paternal allele silent later during seed development. Autorepression of the maternal *MEA* allele is direct and independent of the *MEA*-FIE (FERTILIZATION-INDEPENDENT ENDOSPERM) PcG complex, which is similar to the *E(Z)*-ESC (Extra sex combs) complex of animals, suggesting a novel mechanism. A complex network of cross-regulatory interactions among the other known members of the *MEA*-FIE PcG complex implies distinct functions that are dynamically regulated during reproduction.

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Polycomb group (PcG) genes play an essential role during development of multicellular organisms. PcG genes were first identified in *Drosophila* as mutants causing homeotic transformations because they lead to a deregulation of the *HOX* genes. While an early cascade of transcription factors establishes specific *HOX* gene expression patterns, PcG proteins act in multimeric complexes to maintain the repressed state of their target genes throughout development (Ringrose and Paro 2004). Two distinct PcG complexes have been described in animals, the *Polycomb* Repressive Complex 1 (PRC1) and the Enhancer of Zeste-Extra sex combs [*E(Z)*-ESC] complex (also known as PRC2). These complexes consist of several core components but associate with additional proteins to form distinct subcomplexes in specific tissues (Otte and Kwaks 2003).

[**Keywords:** *Arabidopsis*; genomic imprinting; *MEDEA*; *Polycomb* group; regulation; seed development]

¹These authors contributed equally to this work.

²Present address: Institut Pasteur, Unité de Génétique Moléculaire Murine, 25 rue du Docteur Roux, 75015 Paris, France.

³Corresponding author.

E-MAIL, grossnik@botinst.unizh.ch; FAX 41-(0)44-634-82-04.

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PcG proteins are structurally heterogeneous, but some show functional and structural conservation among *Drosophila*, mammals, and higher plants (Reyes and Grossniklaus 2003; Ringrose and Paro 2004). While no homologs of PRC1 components have been found in the *Arabidopsis* genome, the core components of the *E(Z)*-ESC complex are conserved. There are several variants of the *E(Z)*-ESC complex in plants (Reyes and Grossniklaus 2003; Chanvivattana et al. 2004), the best studied being the *MEA*-FIE complex (Köhler et al. 2003a), which regulates cell proliferation during reproductive development. It consists of the FIS class PcG proteins *MEDEA* (*MEA*), FERTILIZATION-INDEPENDENT ENDOSPERM (*FIE*), and FERTILIZATION-INDEPENDENT SEED2 (*FIS2*), homologs of the *Drosophila* *E(Z)*, *ESC*, and Suppressor of zeste12 [*SU(Z)*12], respectively (Grossniklaus et al. 1998; Luo et al. 1999; Ohad et al. 1999). In addition, animal and plant complexes contain homologs of *Drosophila* p55, a histone-binding WD40 repeat protein (in *Arabidopsis* known as *MSI1*) (Hennig et al. 2003), present in several chromatin-associated complexes.

The *Arabidopsis* *MEA*-FIE complex plays an essential role in sexual reproduction. In flowering plants, meiotic products do not directly differentiate into gametes. Rather, they form multicellular structures, the gametophytes, producing the gametes. The male gametophyte, or pollen, carries two sperm cells that it delivers to the ovule. The female gametophyte, or embryo sac, is enclosed in the ovule and carries the egg and the central cell, which fuse with one sperm each to form the embryo and endosperm, respectively. Mutations impairing components of the *MEA*-FIE complex show pre- and post-fertilization phenotypes associated with abnormal cell proliferation (Köhler and Grossniklaus 2002; Guitton and Berger 2005). In these *fis* class mutants (*mea*, *fie*, *fis2*, and *msi1*), the central cell initiates endosperm development without fertilization. If fertilized, embryo and endosperm derived from *fis* mutant gametes show defects in the regulation of cell proliferation. These phenotypes indicate that the *Arabidopsis* *MEA*-FIE complex shares functional similarities with the corresponding mammalian complex, where mutations in genes encoding its subunits cause aberrant cell proliferation and cancer (Valk-Lingbeek et al. 2004).

Importantly, in *FIS* class genes all mutations lead to maternal-effect seed abortion. For *MEA*, it was shown that the maternal effect is due to the regulation of *MEA* by genomic imprinting. *MEA* is activated prior to fertilization in the embryo sac, and the activity of the maternally inherited allele is maintained in the developing embryo and endosperm, whereas the paternally inherited allele is silent (Kinoshita et al. 1999; Vielle-Calzada et al. 1999). The molecular mechanisms controlling imprinted gene expression at the *MEA* locus are not fully elucidated. Two regulators have been identified that control maternal activation of *MEA* prior to fertilization. DEMETER (*DME*), a DNA-glycosylase, is thought to remove methylated cytosines at the *MEA* locus and acts antagonistically to the DNA-methyltransferase MET1 (Xiao et al. 2003; Choi et al. 2004). However, while there is genetic evidence for an involvement of *DME* and *MET1* in the regulation of *MEA*, it is not known whether these are direct or indirect regulators.

Here we report that *MEA* is involved in the regulation of its own imprinted expression, a feature that has not been described for imprinted genes in animals. Before and after fertilization, *MEA* is required to repress expression of the maternal *MEA^m* allele, while later in seed development, *MEA* keeps the paternally inherited *MEA^p* allele silent. Around fertilization *MEA* interacts with its own promoter, as shown by chromatin immunoprecipitation (ChIP) experiments, suggesting that *MEA* directly controls transcriptional repression of the *MEA^m* allele. Importantly, this autoregulatory function of *MEA* is independent of the *MEA*–*FIE* complex since *MEA* expression is not altered in other *fis* class mutants. This reveals a novel, *MEA*–*FIE* complex-independent function of *MEA*, so far not described for its animal counterpart. We investigated whether other members of the *MEA*–*FIE* complex autoregulate and found a highly dynamic cross-regulatory network. Surprisingly, the *PcG* genes were involved in both negative and positive regulation, suggesting that plant *PcG* proteins may share dual repression/activation functions, directly or indirectly. This indicates that, as its animal counterpart, the formation of the *MEA*–*FIE* complex and related subcomplexes is highly dynamic during plant reproduction.

Results and Discussion

The Polycomb group gene MEA regulates its own imprinted expression

The *MEA* locus is regulated by genomic imprinting where only the maternal allele is actively transcribed after fertilization. Strict maternal expression has been found for the first 52 h after pollination (hap) (Vielles-Calzada et al. 1999), but biallelic expression in the embryo was reported for later stages of seed development (Kinoshita et al. 1999). Afterward, it was discovered that paternal alleles of most genes are not expressed at detectable levels until ≥ 68 hap (Vielles-Calzada et al. 2000). Thus, the question arose whether the allele-specific expression of *MEA* reflected that of an average gene or whether the paternal *MEA^p* allele was persistently down-regulated. For these reasons, we developed a quantitative, allele-specific assay allowing the precise detection of transcripts derived from wild-type *MEA* and mutant *mea* alleles inherited either maternally or paternally (Fig. 1; Supplemental Material). Maternally derived *MEA* transcripts accumulated to their highest level before fertilization and gradually decreased afterward (Fig. 1A,B). During that time, however, no paternal transcript was detectable (Fig. 1A,B). At later seed stages, levels of the maternal allele dropped and were barely detectable above background, while paternal transcripts remained below detection level (data not shown). Our sensitive quantitative assay thus confirmed monoallelic expression of the imprinted *MEA* gene throughout seed development.

Strikingly, our study revealed that mutant *mea* embryo sacs accumulated 45 times more maternal transcripts than wild-type before fertilization (BF) (Fig. 1B), and it persisted after fertilization (7.5 times more transcripts 4 d after pollination [dap]) (Fig. 1B). These findings suggest that transcription of the maternal *mea* allele was derepressed in *mea* mutant embryo sacs and seeds derived thereof. To exclude a possible *cis* effect influenced by the *Ds* transposon present in the *mea-2* allele (Grossniklaus et al. 1998), we analyzed the expression of

Figure 1. *MEA* regulates its own imprinted expression. (A,B) Quantification of maternal and paternal transcripts in ovules and developing seeds derived from wild-type (A) or *mea-2* mutant (B) embryo sacs. Transcripts were quantified before fertilization in entire gynoeceia containing unfertilized ovules (BF) and after fertilization in developing fruits following hand pollination (1–4 dap) (Supplemental Fig. S1). Transcript levels were normalized to *ACTIN11*; average and standard deviation of triplicate quantification are shown. In B, the fold increase of maternal transcripts in *mea* ovules compared with wild-type is indicated for each time point below the graph. (C–E) Expression of a *pMEA::GUS* reporter gene in unfertilized ovules of *MEA/MEA* wild-type plant (C) or *mea-2/MEA* mutant plants (D). (E) The percentage of ovules showing a weak staining as in C or a strong staining as in D was quantified in wild-type and *mea-2/MEA* mutant segregants. (P) Level of significance relative to the difference between the two segregants; (n) number of ovules; (cc) central cell; (ec) egg cell. Bar, 10 μ m. (F–H) Expression of a paternally inherited *pMEA::GUS* reporter gene 5–6 d after crossing to wild-type (F) or *mea-2/mea-2* mutant (G) plants. In H, the percentage showing derepression of the usually silent paternal allele in crosses with wild-type, heterozygous, and homozygous *mea-2* plants is given. (n) Number of seeds analyzed. Bar, 20 μ m.

a reporter gene under the control of the *MEA* promoter (*pMEA::GUS*) in a heterozygous *mea* mutant background. While wild-type segregants heterozygous for the transgene (*MEA/MEA*; *pMEA::GUS*/–) showed weak reporter gene activity (Fig. 1C,E), mutant segregants (*mea/MEA*; *pMEA::GUS*/–) showed 13.5% of ovules with

strong staining in the embryo sac (Fig. 1D,E). Thus, the high levels of maternal *MEA* transcripts observed in a *mea* mutant background and during early development are likely due to negative autoregulation in *trans*. In addition, at later stages (5–6 dap) the paternally introduced *pMEA::GUS* transgene, which is normally silent in a wild-type background (Fig. 1F) was strongly derepressed in a maternal *mea* mutant background (Fig. 1G,H). Thus, *MEA* is also required to maintain the silent state of the paternal *MEA^P* allele, but only at later stages of seed development. However, this late activation of the paternal *MEA^P* allele does not have any phenotypic consequences; i.e., seeds derived from *mea* mutant embryo sacs still abort.

Taken together, these results demonstrate that *MEA* is involved in the regulation of its own imprinted gene expression. The effect is not only allele-specific but also dynamically regulated during seed development. Prior to fertilization and during early seed development, only the maternal *MEA^M* allele is subject to negative autoregulation but not to complete silencing. Then, later during seed development, *MEA* is required to maintain silencing of the paternal *MEA^P* allele. This shows interesting parallels to the EZH2–EED complex, the mammalian counterpart of the *MEA*–*FIE* complex, which is required for paternal silencing of several autosomal imprinted loci (Mager et al. 2003). *MEA* has differential effects on the maternal *MEA^M* and the paternal *MEA^P* allele with respect to developmental stage and the level of repression, indicating that *MEA* function is dynamically regulated during reproductive development.

Maternal *MEA* autoregulation is direct and independent of the *MEA*–*FIE* PcG complex

To determine whether *MEA* directly or indirectly regulates its own expression, we carried out ChIP experiments using the α *MEA* antibody (Köhler et al. 2003b) and tested for enrichment of fragments encompassing the entire *MEA* promoter (Fig. 2A). Given that repression of the paternal *MEA^P* allele is not biologically relevant, we concentrated our analyses on the early phase of reproductive development when repression of the maternal *MEA^M* allele occurs. At this stage, *MEA* is expressed in a very small number of cells (~200 cells per flower), such that very little target chromatin is present in the starting material, making such experiments extremely difficult and variable. Out of six independent ChIP experiments (Supplementary Fig. S2), we found enrichment of *MEA* promoter fragments in four independent chromatin preparations. Fragments #3, #5, and #6 were enriched at least twice in these ChIP experiments (Fig. 2B). These results unequivocally demonstrate an interaction of *MEA* with its own promoter. The interaction occurs in a region of ~800 base pairs (bp) located 700 bp upstream of the transcriptional start site, but binding outside this core region is not excluded as fragments #1 and #9 were also enriched but only once (data not shown).

The finding that *MEA* directly autorepresses its own transcription prior to fertilization prompted us to determine whether this function was mediated by the same *MEA*–*FIE* complex that represses *PHE1* expression during seed development (Köhler et al. 2003b). Clearly, the level of *MEA* transcripts in *fis2*, *fie*, and *msi1* loss-of-function mutants, affecting the other known components of the *MEA*–*FIE* complex, was similar to the level

Figure 2. ChIP experiments show an interaction of *MEDEA* with its own promoter (A) Schematic representation of the *MEA* locus indicating the regions analyzed by PCR after ChIP. The numbers indicate a genomic position relative to the transcriptional start site (arrow) upstream of the ORF of *MEA* (dark shaded gray). The dashed-line box indicates the promoter region where fragments were enriched in the immunoprecipitated fractions in the presence of the α *MEA* antibody. (B) ChIP experiment showing *MEA* interaction with its own promoter. Chromatin was prepared from 1 g of tissue typically consisting of ~1000 wild-type flowers. The gels show PCR products from immunoprecipitated fractions treated with no antibody (lane a), with preimmune serum (lane b), and with α *MEA* antibody (lane c). The complete set of ChIP experiments is shown in Supplementary Figure S2.

in wild-type plants before fertilization and early during seed development (Table 1). We conclude that *MEA* negative autoregulation around fertilization is a function that is independent of the *MEA*–*FIE* complex.

However, additional factors might be involved in *MEA* autoregulation. Before fertilization, the level of wild-type *MEA* transcripts in *mea* heterozygous plants was nine times higher than in wild-type plants (Table 1). Because *MEA* shows gametophytic inheritance and expression (Vielle-Calzada et al. 1999), these transcripts are expected to originate from wild-type embryo sacs and not from the heterozygous *MEA/mea* surrounding maternal tissues. This suggests that in *mea* heterozygous plants, *MEA* is up-regulated in embryo sacs carrying a *MEA* allele (50% of the ovules). It is possible that *MEA* has a function prior to meiosis, although expression is not detectable at this stage of development (Vielle-Calzada et al. 1999). In this case, embryo sacs carrying a wild-type genome would inherit a cytoplasm depleted in a factor normally involved in repressing *MEA*. Depletion of this factor might in turn result from haplo-insufficiency of *MEA* prior to meiosis. Alternatively, *mea* may have a subtle effect in heterozygotes that leads to the accumulation of epigenetic changes, which may affect regulators of *MEA* expression. Such secondary epigenetic effects have been observed for mutations in the *DECREASE IN DNA METHYLATION1* (*DDM1*) gene (Kakutani et al. 1996). Consistent with this hypothesis, derepression of *MEA* is even stronger in homozygous *mea* mutant inbred lines (Supplementary Table S1). Finally, the *Ds* transposon might cause incomplete pairing at meiosis. Similar to meiotic silencing of unpaired DNA in *Neurospora crassa* (Shiu et al. 2001), unpaired DNA at the *MEA* locus could trigger an epigenetic modification rendering the *MEA^M* allele in wild-type embryo sacs insensitive to autorepression.

Taken together, we provide evidence that *MEA* directly represses its own imprinted expression around fer-

Table 1. Auto- and cross-regulation among the PcG genes MEA, FIS2, FIE, and MSI1

	Mutant	Transcript	BF	1 dap	2 dap	3 dap
Autoregulation	<i>mea-1/+</i>	<i>MEA</i>	↗ 9× ***	↗ 11× ***	↗ 4× **	≈
		<i>mea-1</i>	↗ 17× ***	↗ 16× ***	↗ 7.5× ***	≈
	<i>fis2-1/+</i>	<i>fis2, FIS2</i>	≈	≈	≈	≈
	<i>fie-2/+</i>	<i>fie, FIE</i>	≈	↘ 4× **	≈	≈
	<i>msi1-2/+</i>	<i>msi1, MSI1</i>	≈	≈	≈	≈
Cross-regulation	<i>mea-1/+</i>	<i>FIS2</i>	↘ 3.5× **	≈	≈	≈
		<i>FIE</i>	↘ 5× *	≈	≈	↘ 1.5× **
		<i>MSI1</i>	↘ 5× *	≈	≈	↘ 1.5× **
	<i>fis2-1/+</i>	<i>MEA</i>	≈	≈	≈	≈
		<i>FIE</i>	↘ 2.5× **	≈	≈	↗ 1.5× **
		<i>MSI1</i>	↘ 3× **	≈	≈	≈
	<i>fie-2/+</i>	<i>MEA</i>	≈	≈	≈	≈
		<i>FIS2</i>	≈	↘ 4× **	≈	≈
		<i>MSI1</i>	≈	↘ 2.5× *	≈	≈
	<i>msi1-2/+</i>	<i>MEA</i>	≈	≈	≈	≈
		<i>FIS2</i>	≈	≈	≈	≈
		<i>FIE</i>	≈	≈	≈	≈

Transcript levels were measured in at least three biological replicates (independent sample collection and cDNA preparation) (Supplementary Table S1) from emasculated gynoecea (BF) or young siliques (1–3 dap) using quantitative real-time RT-PCR assays. The assay did not discriminate between transcripts derived from wild-type and mutant alleles, except for *mea-1* and *MEA*, which were detected using two specific assays. Relative transcript levels were normalized to the levels of *ACT11*. The fold increase (↗) or decrease (↘) compared with wild-type levels is indicated; the level of significance was assessed using a *t*-test: (*) 0.01 < *P* < 0.05; (**) 0.001 < *P* < 0.01; (***) *P* < 0.001; (≈) *P* > 0.05, no significant difference. The values of transcript levels relative to wild-type levels are provided in Supplementary Table S1.

tilization. Transcriptional repression is mediated by an interaction of MEA with its own promoter and, importantly, does not require the MEA–FIE complex. All known PcG complexes in *Arabidopsis* involve FIE, the homolog of ESC. FIE is encoded by a unique gene and serves as platform for complex assembly (Köhler and Grossniklaus 2002; Reyes and Grossniklaus 2003), such that in *fie* mutants no PRC2-like complexes are formed. Thus, the autoregulatory function of MEA must involve novel, possibly non-PcG, partners that remain to be identified.

Auto- and cross-regulation among the PcG genes MEA, FIS2, FIE, and MSI1

To gain insight into potential regulatory interactions among members of the MEA–FIE complex, we investigated their expression profiles around fertilization in wild-type and loss-of-function mutants using quantitative RT-PCR.

In wild-type ovules *MEA*, *FIS2*, and *MSI1* transcripts are accumulated at their highest level before fertilization and gradually decrease after fertilization, while *FIE* transcript levels increase transiently just after fertilization (Fig. 3A). The relative abundance of transcripts varies drastically between the *FIS* genes (Fig. 3B) and might be explained, in part, by their different expression patterns in ovules: Only *MEA* and *FIS2* are specific to the embryo sac and the two fertilization products, while *FIE* and *MSI1* expression extends to some maternal tissues (Grossniklaus et al. 1998; Vielle-Calzada et al. 1999; Luo et al. 2000; Spillane et al. 2000; Köhler et al. 2003a).

In contrast, transcript levels of the *FIS* genes were drastically modified in *mea*, *fis2*, and *fie* but not *msi1* mutants, revealing both auto- and cross-regulatory feedback loops (Table 1). While *fis2* and *msi1* mutations had no effects on their own expression, the *fie* mutation

caused a severe reduction of its own transcript levels after fertilization, indicating direct or indirect autoregulation (Table 1). Before fertilization, a significant 3.5-fold to fivefold reduction of *FIS2*, *FIE*, and *MSI1* transcripts was observed in *mea* heterozygous plants, and a similar reduction of *FIE* and *MSI1* was found in the *fis2* mutant. The reduction did not persist after fertilization, and transcripts levels were close to wild-type in *mea* and *fis2* heterozygotes at 1 dap. By contrast, in the *fie* mutant *FIS2* and *MSI1* levels were not altered before fertiliza-

Figure 3. Expression profiles of *MEA*, *FIS2*, *FIE*, and *MSI1*. (A) Relative transcript levels in wild-type gynoecea (BF) or developing siliques (1, 2, and 3 dap). The levels were normalized to 1 before fertilization. The average of three biological replicates is plotted on the graph, and the standard deviation is shown. (B) Relative transcript abundance at the BF time point. The ratios are expressed relative to the level of *FIS2*, which was normalized to 1.

tion, whereas a significant 2.5-fold and fourfold decrease was observed just after fertilization, respectively. In all mutants the levels of *FIS2*, *FIE*, and *MSI1* transcripts were back to normal at 2 dap. Moderate but distinct effects on *FIE* and *MSI1* expression levels were observed again in *mea* and *fis2* heterozygotes at 3 dap.

Down-regulation in *fis* mutants could be due to an excess-of-silencing mediated by mutant forms of PcG proteins, as described for the *Tritorax-mimick* mutant allele of *E(z)* in *Drosophila* (LaJeunesse and Shearn 1996; Bajusz et al. 2001). This seems unlikely, however, because the lesions in the *mea-1*, *fis2-1*, and *fie-2* alleles used in this study (Grossniklaus et al. 1998; Luo et al. 1999; Ohad et al. 1999) cause truncation in the SET, VEFS, and WD40 domains of MEA, FIS2, and FIE, respectively, which are essential to the formation of a functional PcG complex (Denisenko et al. 1998; Cao and Zhang 2004; Chanvivattana et al. 2004; Yamamoto et al. 2004; Nekrasov et al. 2005). Instead, it is likely that MEA, FIS2, and FIE directly or indirectly activate FIS2, FIE, and MSI1 expression.

Our results clearly show that expression of the *FIS* genes is modulated around fertilization by auto- and cross-regulatory interactions (Fig. 4). Interestingly, *FIE* autoregulation and MEA positive regulation of *FIE* exactly reflects the situation in *Drosophila*, where *ESC* autoregulates and *E(Z)* positively regulates *ESC* (Ali and Bender 2004). Positive cross-regulation extends to other members of the MEA–FIE complex, except MSI1, which does not influence MEA, FIS2, and FIE expression, although these proteins in turn regulate MSI1 expression.

Figure 4. Complex regulatory interactions among the *FIS* class PcG genes. (A) MEA regulates its own imprinted gene expression. Schematic representation of the known regulators of imprinted MEA expression. Direct interactions are shown as solid lines, and interactions that are either direct or indirect are shown as stippled lines. Before fertilization, the maternal MEA^m allele is activated through antagonistic interactions of DME and MET1 (Xiao et al. 2003) and kept at a low expression level by direct autorepression. After fertilization, the paternal MEA^p allele is maintained silent through MEA-mediated repression and activity of the chromatin remodeling factor DDM1, based on genetic evidence (Vielle-Calzada et al. 1999). (B) Genetic regulation of the *FIS* class PcG genes involves positive auto- and cross-regulations. Only MEA and FIE autoregulate. MEA autorepression is direct but independent of the other components of the MEA–FIE complex. The mechanism of FIE autoregulation is unknown but is also independent of the MEA–FIE complex. A comparison of regulatory interactions before and after fertilization illustrates the highly dynamic nature of PcG regulation.

The physiological significance of these complex regulatory interactions for seed development remains to be elucidated. Most likely they ensure the fine-tuning of the MEA–FIE complex protein levels and may regulate their dynamic participation in distinct variants or subcomplexes during development.

The positive feedback loops in this cross-regulatory network are noteworthy (Fig. 4). Two possibilities arise to explain the activating function of MEA, FIS2, and FIE. Possibly, they could down-regulate the expression of transcriptional repressors of FIS2, FIE, and MSI1. Alternatively, they could take part in novel activating complexes. The latter hypothesis is based on antagonistic repression/activation properties reported for several PcG proteins in *Drosophila*. *E(Z)* and other PcG genes have been reclassified as *Enhancers of trithorax and Polycomb* (ETP) on the basis that some mutant alleles could not only enhance *Polycomb* but also *trithorax* homeotic defects (LaJeunesse and Shearn 1996; Gildea et al. 2000; Bajusz et al. 2001). Thus, MEA, FIS2, and FIE may be plant ETPs that, as *Drosophila* ETPs, take part in distinct complexes with transcriptional repression and activation properties (Brock and van Lohuizen 2001).

In summary, our studies have not only identified MEA as a novel direct regulator of its own imprinted gene expression, but have also highlighted the complex and highly dynamic nature of auto- and cross-regulatory interactions among the *FIS* genes. Clearly, MEA has distinct effects on gene regulation at various stages of reproductive development, likely reflecting a dynamic composition of MEA-containing complexes. This dynamic behavior may indicate that, in plants, PcG repression is not as persistent as it is in animals but is often released upon external or internal stimuli, such as fertilization.

Material and methods

Plant material

Wild-type plants were *Arabidopsis thaliana* (L.) Heynh. accession Landsberg (*erecta* mutant, *Ler*) and accession C24. The mutant alleles used were *mea-1*, *mea-2* (Grossniklaus et al. 1998), *fis2-1* (Luo et al. 1999), *fie-2* (Ohad et al. 1999), and *msi1-2* (Guitton et al. 2004). The *mea-1* and *mea-2* mutant plants were selected on kanamycin, while the others were genotyped using SSLP or dCAPs markers (Supplemental Material). Establishment of the *pMEA::GUS* transgenic lines and staining procedures are described in Supplemental Material.

Quantitative RT-PCR

The accumulation of MEA, *mea*, FIS2, FIE, and MSI1 transcripts were measured using quantitative real-time RT-PCR as described by Köhler et al. (2003b). Details can be found in the Supplemental Material and Supplementary Figure S3. In brief, quantitative analyses of transcript levels were carried out using Sybr Green or Taqman real-time PCR assays (Applied Biosystem). Three PCR replicates were performed for each cDNA sample, and the specificity and amount of the unique amplification product were determined according to the manufacturer's instructions (Applied Biosystems). For measuring maternal and paternal MEA transcripts, we used the *mea-1* and *mea-2* mutant alleles (Grossniklaus et al. 1998), which produce mutant transcripts containing transposon sequences (Supplementary Fig. S3), allowing specific detection and discrimination from wild-type MEA transcripts. We applied this assay to distinguish maternal and paternal transcripts in a set of reciprocal crosses between wild-type and homozygous *mea-2* (Fig. 1) or in selfed *mea-1* heterozygous lines (Table 1). In all experiments, transcript levels were normalized to the level of *ACT11*, which is expressed in the gametophyte and zygotic products of the seed (embryo and endosperm) but not in the surrounding maternal tissues (Huang et al. 1997). Beyond 4 dap, *ACT11* levels decrease and cannot be used for normalization (data not shown).

ChIP and PCR

Six independent experiments were carried out using ~1000 flowers and young siliques around fertilization (1 g tissue). We estimated that the starting material encloses ~50,000 female gametophytes (where MEA is expressed) containing 50–150 fg target DNA (for a 1-kb fragment) before immunoprecipitation. ChIP was performed according to Köhler et al. (2003b) with one minor modification: Reverse cross-linking was carried out overnight at 65°C.

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Note added in proof

Recently, two other publications reported regulation of the paternal *MEA^p* allele by the MEA–FIE complex and showed that paternal silencing is correlated with histone methylation on H3K27 (Gehring et al. 2006; Jullien et al. 2006).

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