Identification of a DNA methylation-independent imprinting control region at the *Arabidopsis MEDEA* locus

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Genomic imprinting is exclusive to mammals and seed plants and refers to parent-of-origin-dependent, differential transcription. As previously shown in mammals, studies in *Arabidopsis* have implicated DNA methylation as an important hallmark of imprinting. The current model suggests that maternally expressed imprinted genes, such as *MEDEA* (*MEA*), are activated by the DNA glycosylase DEMETER (DME), which removes DNA methylation established by the DNA methyltransferase MET1. We report the systematic functional dissection of the *MEA cis*-regulatory region, resulting in the identification of a 200-bp fragment that is necessary and sufficient to mediate *MEA* activation and imprinted expression, thus containing the imprinting control region (ICR). Notably, imprinted *MEA* expression mediated by this ICR is independent of DME and MET1, consistent with the lack of any significant DNA methylation in this region. This is the first example of an ICR without differential DNA methylation, suggesting that factors other than DME and MET1 are required for imprinting at the *MEA* locus.

[*Keywords*: *Arabidopsis*; *DEMETER*; DNA methylation; genomic imprinting; *MEDEA*; imprinting control region]

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Genomic imprinting is a form of epigenetic gene regulation, which leads to the differential expression of an allele according to its parent of origin. Its discovery dates back to 1970, when Kermicle (1970) described the maternal effect of the *R* gene, which controls maize kernel coloration. Later, an analogous phenomenon was identified in mice when pronuclear transplantation experiments revealed that both maternal and paternal genomes were required to achieve normal development (McGrath and Solter 1984; Surani et al. 1984). Imprinted genes encode for diverse proteins that function in growth and cellular proliferation, typically in extraembryonic tissues involved in nourishing the newly developing organism; i.e., the placenta in mammals and the endosperm in plants (Grossniklaus 2005; Feil and Berger 2007). The endosperm results from double fertilization in angiosperms: While one sperm cell fertilizes the egg cell, giving rise to the embryo, the second sperm cell fuses with the central cell, leading to the development of the endosperm (Maheshwari 1950). Genomic imprinting in mammals and seed plants evolved independently, but likely in response to similar selective pressures that maintain a fine balance between competing interests of the maternal and paternal genomes in resource allocation (Haig and Westoby 1989; Moore and Reik 1996; Messing and Grossniklaus 1999).

Although some imprinted plant genes are also expressed in the embryo, most show preferential expression in the triploid endosperm, and some of them are essential for seed development (for review, see Raissig et al. 2011). *MEDEA* (*MEA*) and *FERTILIZATION-INDEPENDENT SEED2* (*FIS2*) are maternally expressed genes encoding evolutionary conserved *Polycomb* group (PcG) proteins (Grossniklaus et al. 1998; Luo et al. 1999). Plant PcG proteins form several variants of multiprotein complexes that maintain a silenced state of gene expression over many cell divisions through histone modifications (Pien and Grossniklaus 2007). The MEA–FIE (FERTILIZATION-INDEPENDENT ENDOSPERM) complex, which regulates cell proliferation in the endosperm and embryo, contains the PcG proteins MEA, FIS2, FIE, and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) (Ohad et al. 1999; Luo et al. 2000; Spillane et al. 2000; Köhler et al. 2003a). Mutations in any of these *FIS*

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class genes (*mea*, *fie*, *fis2*, and *msi1*) lead to maternal-effect seed abortion (for review, see Grossniklaus et al. 2001), which, in the case of *MEA* and *FIS2*, is due to their maternal-specific expression (Kinoshita et al. 1999; Vielle-Calzada et al. 1999; Jullien et al. 2006b). To date, *PHERES1* (*PHE1*), which is directly regulated by MEA, represents the only well-studied paternally expressed imprinted gene in plants (Köhler et al. 2003b, 2005). While *MEA* and *FIS2* are required for normal seed development (Grossniklaus et al. 1998; Luo et al. 1999), and *PHE1* plays a role in seed abortion in hybrids (Josefsson et al. 2006), two other maternally expressed genes that were reported to be imprinted, *FLOWERING WAGENINGEN* (*FWA*) and *AGAMOUS-LIKE 36* (*AGL36*), are not essential for seed development (Kinoshita et al. 2004; Shirzadi et al. 2011). Recently, several studies using allele-specific RNA profiling of the seed transcriptome describe many novel candidate imprinted genes in *Arabidopsis* (Gehring et al. 2011; Hsieh et al. 2011; Wolff et al. 2011), rice (Luo et al. 2011), and maize (Waters et al. 2011; Zhang et al. 2011). Yet, little is known concerning their role during seed development or their allele-specific regulation.

In contrast, the molecular mechanism underlying the maternal monoallelic expression of *MEA*, *FIS2*, and *FWA*, which results from genomic imprinting (for review, see Grossniklaus 2005), has been studied in some detail. Imprinting of all three loci results from a combination of maternal allele activation and paternal allele silencing. DNA and histone methylation function as epigenetic marks to distinguish maternal and paternal alleles, with DNA methylation playing a critical role in the regulation of all three loci (Vielle-Calzada et al. 1999; Luo et al. 2000; Kinoshita et al. 2004; Jullien et al. 2006b). The current model for imprinting control of *FIS2* and *FWA* involves repressive DNA methylation of both parental alleles by the maintenance DNA methyltransferase MET1 throughout vegetative development. The silencing of the paternal *MEA* allele, however, depends on repressive histone H3 Lys 27 methylation (H3K27me) mediated by a vegetatively acting PcG complex (Jullien et al. 2006a). During male gametogenesis, paternal allele silencing is maintained by MET1 for *FIS2* and *FWA* but by the PcG protein FIE at the paternal *MEA* allele, since in MET1-deficient pollen, the paternal *MEA* allele is not derepressed (Gehring et al. 2006; Jullien et al. 2006a,b). In contrast, during female gametogenesis, the DNA glycosylase DEMETER (DME) removes maternal DNA methylation at all three loci, which results in expression of the maternal allele in the central cell and, subsequently, during seed development (Choi et al. 2002; Kinoshita et al. 2004; Gehring et al. 2006; Jullien et al. 2006b). This demethylation process also involves a histone chaperone, illustrating the interplay of DNA methylation and chromatin level regulation (Ikeda et al. 2011).

In addition to the shared regulation of imprinting at the *FIS2* and *FWA* loci, additional mechanisms appear to operate at the *MEA* locus: *MEA* is expressed in both the embryo and endosperm, and paternal *MEA* allele expression has not been detected during early seed development, suggesting that it is imprinted in both fertilization products at these stages, at least in some accessions (Vielle-Calzada et al. 1999; Luo et al. 2000; Spillane et al. 2007; Raissig et al. 2011). Thus, it is currently unknown how the maternal *MEA* allele is activated in the embryo in the absence of DME activity, which is thought to be restricted to the central cell (Choi et al. 2002). Nevertheless, maternal *MEA* allele activation in the central cell by DME has been the main focus of imprinting regulation in *Arabidopsis*, and possible DME target regions at the *MEA* locus have been identified: The *AtREP2* helitron, CG sites 3 kb and 500 bp upstream of the *MEA* coding region, and the MEA-intergenic subtelomeric repeat (ISR) (Cao and Jacobsen 2002) downstream from the *MEA* coding region were shown to be methylated (Xiao et al. 2003). Indeed, DME establishes allele-specific hypomethylation of the maternal *MEA* allele at the −500-bp region and the MEA-ISR, suggesting that these regions control *MEA*-imprinted expression via their methylation status (Gehring et al. 2006). However, *Arabidopsis* accessions lacking the MEA-ISR remain imprinted at the *MEA* locus (Spillane et al. 2004), and the methylation status of the −500-bp region is not only controlled by DME, but varies depending on the accession; i.e., this region is unmethylated in the Landsberg *erecta* (L*er*) accession, despite *MEA* being imprinted in L*er* (Spillane et al. 2004; Gehring et al. 2006; Schoft et al. 2011). Taken together, this challenges DME as the regulator of imprinted *MEA* expression and raises the question of the actual *cis*-regulatory element for *MEA* imprinting.

Here we report on a minimal 200-base-pair (bp) fragment from the *MEA cis*-regulatory region that faithfully recapitulates *MEA*-like expression and functionally complements the *mea* mutation. Hence, it contains all of the necessary elements for transcriptional activation and imprinting control. We show that activation by *DME* is not mediated by this 200-bp fragment, thereby uncoupling maternal activation by DME from the imprinting control region (ICR). Genetic analysis of seed abortion indicated that DME and MET1 are only indirectly involved in *MEA* imprinting regulation. Maternally, *dme*-induced seed abortion could not be rescued by a functional *MEA* transgene; paternally, rescue of *mea*-induced seed abortion by *met1* mutant pollen was not linked to a functional paternal *MEA* allele. As suggested previously (Gehring et al. 2006), allele-specific expression analysis showed that paternal *MEA* silencing is independent of MET1, consistent with the lack of significant methylation in the *MEA*-ICR. We propose a new model of *MEA* imprinting, in which DME and MET1 affect higher-order chromatin structure through targeting of transposon-related sequences but are not directly involved in the regulation of *MEA* imprinting.

Results

Cis-*activating regions and ICRs reside in the 200-bp* MEA *promoter*

In order to identify the minimal *cis*-regulatory region for imprinted *MEA* expression, we undertook a systematic deletion analysis of the *MEA cis*-regulatory sequences (Fig. 1A). The −4-kb *MEA* upstream sequence, which was

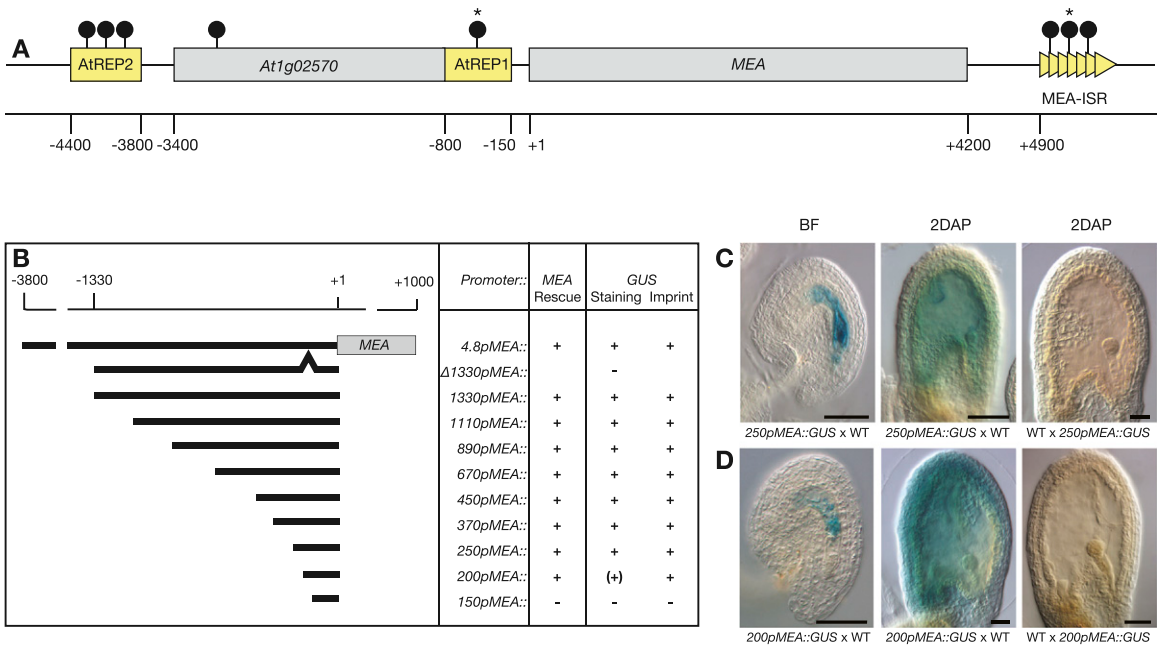

Figure 1. *MEA* promoter dissection. (*A*) The *MEA* locus contains two helitron transposons, *AtREP2* and *AtREP1*, 5′ of the translational start site and a tandem repeat region, termed MEA-ISR, 3′of the gene. *At1g02570* resides in the formerly designated *MEA* promoter (see also Supplemental Fig. S1). Numbers are relative to the translational start site. (Gray boxes) Genes; (yellow boxes) transposons and repeats; (arrowheads) 182-bp direct repeats; (lollipops) sites of DNA methylation as reported (Xiao et al. 2003; Gehring et al. 2006); (stars) hypomethylation of maternal *MEA* endosperm alleles at 7–9 d after pollination (DAP). (*B*) The *4.8pMEA::MEA* transgene contains 3.8 kb of *MEA* upstream sequence fused to *MEA* cDNA and was shown to complement the *mea*-induced seed abortion phenotype (Makarevich et al. 2006). The *4.8pMEA::GUS* transgene was previously described (Spillane et al. 2004) and contains 3.8 kb of *MEA* upstream sequence plus 1 kb of *MEA* coding region. The other transgenes consist of 1330-bp to 150-bp *MEA* promoter sequence fused to *MEA* genomic DNA (*pMEA::MEA*) or the bacterial *uidA* reporter gene (*pMEA::GUS*). In the *Δ1330pMEA::GUS* transgene, the region between the −200-bp and −150-bp *MEA* upstream sequence is deleted. Plus signs [+] indicate positively tested for rescue, staining, or imprinting; minus signs [−] indicate negatively tested for rescue, staining, or imprinting; the plus sign in parenthesis [(+)] indicates deviation from *MEA*-like GUS staining; and empty fields indicate that the corresponding promoter fusion was not tested. (*C*,*D*) Expression of a *250pMEA::GUS* transgene (*C*) and a *200pMEA::GUS* transgene (*D*). The transgenes were reciprocally crossed to L*er* wild-type plants. Maternal GUS activity is detected with both transgenes before fertilization (BF) and 2 DAP. No paternal GUS activity is detected. For detailed GUS expression analysis, see Supplemental Figure S2. Bar, 50 μm.

shown to confer imprinted expression (Spillane et al. 2004), contains the previously unidentified gene *At1g02570*. It was recently annotated based on expressed sequence tags found in transcription profiling studies and encodes a protein of unknown function (Schmid et al. 2003; Castelli et al. 2004). As it resides between regions implicated in *MEA* regulation, we analyzed expression of *At1g02570* by RT–PCR before and after fertilization. We found no expression during early seed development when *MEA* is expressed (Supplemental Fig. S1), suggesting that this gene does not share regulatory *cis*-elements with *MEA*.

Using the previously described *4.8pMEA::GUS* reporter construct, which comprises 3.8 kb of upstream and 1 kb of coding region of the *MEA* gene (Spillane et al. 2004), successive 5′ deletions were introduced, leading to fragment lengths ranging from 1330 bp to 150 bp of *MEA* *cis*-regulatory sequence. We constructed transcriptional fusions to the *Escherichia coli uidA* gene (*pMEA::GUS*), encoding β-glucuronidase (GUS), and to *MEA* genomic DNA (*pMEA::MEA*) for expression and functional analyses, respectively (Fig. 1B). Several independent primary transformants for each transgene were recovered and scored for *MEA*-like expression (Supplemental Tables S1, S2). Only transgenic lines containing a single copy of the insertion, as determined by Southern blot analysis, were chosen for experiments investigating *MEA* imprinting regulation (data not shown).

We studied maternal *GUS* expression from before fertilization until 4 d after pollination (DAP), corresponding to the globular stage of embryo development (Fig. 1C; Supplemental Fig. S2A,C,D). The plant line harboring the *4.8pMEA::GUS* transgene was used as a reference, its GUS-staining pattern reflecting *MEA* expression (Supplemental Fig. S2A). *pMEA::GUS* transgenes with 1330 bp to 250 bp of *MEA* *cis*-regulatory sequences resulted in GUS-staining patterns that were indistinguishable from the *MEA*-like reference pattern: GUS activity was first detected in gynoecia before fertilization, with the entire embryo sac displaying a strong blue staining. After fertilization, GUS activity was found in the embryo, in the free nuclei in the peripheral endosperm, and at the chalazal cyst region of the endosperm. At 4 DAP, weak

GUS activity was detected in globular stage embryos and at the chalazal pole in the endosperm. Therefore, the minimum element necessary to confer *MEA*-like expression resides in the 250-bp *MEA* upstream sequence.

Reducing the fragment length further to only 200 bp of the upstream sequence resulted in a slightly different GUS-staining pattern, which extended into the surrounding sporophytic endothelium (Fig. 1D; Supplemental Fig. S2F,G). The altered expression observed with the *200pMEA::GUS* indicates that a sporophytic repressor-binding site is located between −250 and −200 bp. We observed no GUS activity in plants with the *150pMEA::GUS* transgene and therefore proposed that the 50-bp fragment, which extends from −200 bp to −150 bp, is required for *cis*-activation of *MEA* expression. Indeed, deletion of this 50 bp in the context of the *1330pMEA::GUS* transgene resulted in a loss of expression in all independent primary transformants analyzed (Fig. 1B; Supplemental Table S1). The 50-bp fragment alone did not result in any detectable expression when fused to a *min35S::GUS* transgene (data not shown), indicating that this fragment is necessary but not sufficient for *cis*-activation of *MEA*.

To test for potential loss of imprinting of the reporter transgenes, we reciprocally crossed plants containing the different *pMEA::GUS* transgenes and looked for possible paternal *pMEA::GUS* expression. All reporter transgenes showing *MEA*-like expression were active only when inherited from the mother (Fig. 1C,D; Supplemental Fig. S2A,C,D,F,G), whereas paternally inherited transgenes were silent (Fig. 1C,D; Supplemental Fig. S2B,E,H). Thus, a *cis*-regulatory fragment as short as 200 bp is able to confer imprinted expression to a *GUS* reporter gene, suggesting the presence of an ICR within this fragment.

The 200-bp fragment mediates functional MEA *expression rescuing seed abortion*

In order to functionally test the *pMEA* fragments, we investigated seed abortion in *mea/MEA* plants transformed with *pMEA::MEA* transgenes. Heterozygous *mea/MEA* mutant plants show 50% seed abortion, and all seeds carrying a maternally inherited *mea* mutation abort irrespective of the paternal contribution (Grossniklaus et al. 1998). We scored seed abortion in transgenic *mea/MEA* plants to look for complementation of the *mea*-induced 50% seed abortion phenotype. In all primary transformants except the ones carrying the *150pMEA::MEA* transgene, we found rescue of the *mea* mutant phenotype illustrated by reduced seed abortion frequencies (Supplemental Table S2). Thus, the 200-bp *cis*-regulatory fragment is necessary and sufficient for functional expression of *pMEA::MEA* transgenes, recapitulating the results with the *pMEA::GUS* transgenes at the functional level.

Taken together, our systematic analysis has uncovered a 200-bp minimal fragment of the *MEA cis*-regulatory region that contains the elements necessary and sufficient for transcriptional activation and imprinting control. An additional element between −250 bp and −200 bp is needed to repress sporophytic expression in the ovule. Thus, we used the *250pMEA::GUS* transgene, reflecting *MEA*-like expression, to investigate *MEA* imprinting control in combination with allele-specific expression analyses of the endogenous *MEA* locus.

MEA-*ICR sequence elements are found upstream of or downstream from other potentially imprinted loci*

We investigated whether sequence elements from the *MEA*-ICR were also present at other potentially imprinted loci. To this aim, we performed a WU-BLAST analysis (http://www.arabidopsis.org/wublast/index2.jsp) of the entire *250pMEA* promoter sequence and of the promoter sequence required for proper *MEA*-like expression (100-bp element between −250 and −150 from the *MEA* start codon) against 3 kb of upstream and downstream sequences of all TAIR10 loci (http://www.arabidopsis.org/wublast/index2.jsp). We then compared the output (684 loci) with all potentially imprinted genes that were recently reported (Gehring et al. 2011; Hsieh et al. 2011; McKeown et al. 2011; Wolff et al. 2011). Interestingly, we found that 15 of these recently published imprinted candidate genes do have conserved sequences upstream of or downstream from the respective gene, suggesting that some *MEA*-ICR sequence elements might be conserved between genes regulated by genomic imprinting (see Supplemental Table S3). A permutation test using 1000 randomized gene samples (n = 684) showed that the probability of finding >14 of the recently described imprinted candidate genes by chance is only $P = 0.051$.

In addition, we performed a motif analysis of the *MEA*-ICR and the putative regulatory sequences of the six imprinted candidate genes with the highest similarity scores (i.e., the smallest *P*-values) using the PLACE database (Higo et al. 1999). Interestingly, we found that GT1-binding sites and DOF-binding elements, both of which are abundant in the *MEA*-ICR (nine and five sites, respectively), were also present in the putative regulatory sequences of all six imprinted candidate genes analyzed (Supplemental Table S4). Surprisingly, a pollen-associated binding element, which we speculate might be involved in recruiting repressors to the paternal allele in the male gametophyte, was also found in all of these sequences. An overview of the identified motifs, including other expected *cis*-regulatory elements such as TATABOX5, GATABOX, and a poly-A signal box, is shown in Supplemental Table S4. However, none of these six candidate imprinted genes was analyzed for regulation by *MET1* or *DME*, such that we have no information on their dependence on DNA methylation. Expression of three of the candidates was analyzed in a *fie* mutant background (*At3g19160*, *At2g18880*, and *At4g29650*) (Wolff et al. 2011), but disruption of PRC2 (Polycomb-repressive complex 2) had no effect on their expression.

Taken together, these bioinformatic analyses showed that some sequence elements of the *MEA*-ICR are conserved in putative regulatory sequences of other imprinted loci. Yet these motifs constitute only a small part of the conserved region, as most of the similarity is based on the high A+T content of the *MEA*-ICR (70%). Nevertheless, the imprinted candidate genes with the highest similarity

do share common motifs, such as GT1-binding sites and DOF-binding elements, possibly reflecting conserved regulatory mechanisms.

The MEA*-ICR mediates activation of maternal* MEA *expression independent of DME*

Allele-specific demethylation of the maternal *MEA* allele by DME in the central cell was proposed to selectively activate the maternal *MEA* allele, whereas the paternal *MEA* allele remains silenced (Gehring et al. 2006). However, the *250pMEA::GUS* transgenes are maternally active and paternally silent even though they lack the −500-bp region targeted by DME-dependent demethylation. To elucidate the impact of *DME* on *MEA*-imprinted expression, we analyzed the maternal activity of two *pMEA::GUS* transgenes in the *dme-4* mutant background (Guitton et al. 2004). We crossed plants homozygous for a single locus of either the *4.8pMEA::GUS* or *250pMEA::GUS* transgene to *dme-4/DME* plants and analyzed the progeny for maternal GUS activity. All F1 plants are hemizygous for the *pMEA::GUS* transgene, and half of them are *dme-4/DME* or *DME/DME*, respectively. F1 plants segregating the *dme-4* mutation were emasculated and analyzed for their GUS-staining pattern before fertilization.

In *DME* wild-type plants hemizygous for either *250pMEA::GUS* or *4.8pMEA::GUS*, we observed 50% and 47% GUS staining in unfertilized ovules, respectively, consistent with Mendelian inheritance of the *pMEA::GUS* transgenes by one-half of the female gametophytes (Fig. 2A,B). In plants hemizygous for the *pMEA::GUS* transgene and heterozygous *dme-4/DME*, one-fourth of the ovules are predicted to inherit both the wild-type *DME* allele and the *pMEA::GUS* transgene, whereas one-fourth will inherit the mutant *dme-4* allele along with the *pMEA::GUS* transgene. If *DME* is a direct activator of maternal *MEA* allele expression, we would expect to see only 25% GUS-staining ovules in *dme-4/DME* plants. Indeed, we found a significant reduction ($P = 0.0003$) from 47% to 34% GUS-staining ovules in *dme-4/DME* plants with the *4.8pMEA::GUS* transgene (Fig. 2A,B), suggesting that the *4.8pMEA::GUS* transgene was partly subject to *DME*-dependent repression. In plants hemizygous for *250pMEA::GUS* and *dme-4/DME*, we obtained 46% GUS-staining ovules (Fig. 2A,B). This is not significantly different ($P = 0.9667$) from the 50% GUS staining found in the *DME* wild-type background, suggesting that all of the ovules inheriting the *dme-4* mutation expressed *250pMEA::GUS*.

Thus, *DME* activation of maternal transgene expression before fertilization is dependent on the *MEA* promoter length, which is likely due to the presence of the *AtREP2* helitron 4 kb upstream of *MEA*. This is supported by a previous study, which demonstrated that *4.2pMEA::GUS* and *4.2pMEA::GFP* transgenes containing 450 bp of *AtREP2* are only active when a maternal wild-type *DME* copy is provided (Choi et al. 2002). Our *4.8pMEA::GUS* transgene, containing 3.8 kb of *MEA* upstream sequence with only 100 bp of *AtREP2*, is partially dependent on *DME* activation, whereas maternal activation of the *250pMEA::GUS* transgene is completely independent of *DME* function. As the *250pMEA::GUS* transgene shows exclusive maternal expression, we conclude that DME is not required for imprinting control beyond the native genomic context; i.e., DME is not targeted to the *MEA*-ICR for activation of maternal *MEA* expression.

The MEA*-ICR mediates paternal transgene silencing by maternal MEA*

The *MEA* promoter analysis revealed the existence of a *MEA*-ICR in the 200-bp fragment. Subsequently, we could show that maternal *MEA* allele activation by DME is not targeted to the *MEA*-ICR on the maternal allele. Therefore, we sought to test whether the previously suggested mechanism for paternal *MEA* allele silencing, involving DNA and histone methylation (Gehring et al. 2006; Jullien et al. 2006a,b), is mediated by the *MEA*-ICR.

The MEA–FIE complex represses the *MEA* paternal allele via deposition of repressive H3K27 dimethylation (H3K27me2), which has been found in a region close to the *MEA* transcriptional start site (Gehring et al. 2006). We asked whether the *MEA*-ICR still responds to repression by the MEA–FIE complex. Therefore, we analyzed *GUS* expression in reciprocal crosses of plants homozygous for the *250pMEA::GUS* transgene and homozygous for *mea-1*. Pollination of female plants homozygous for the *250pMEA::GUS* transgene with *mea-1* mutant pollen (Fig. 3A,B) resulted in the same maternal GUS-staining pattern as in females pollinated with wild-type pollen (Supplemental Fig. S2C). Although the *250pMEA::GUS* transgene is imprinted and paternally not expressed after fertilization of a wild-type ovule

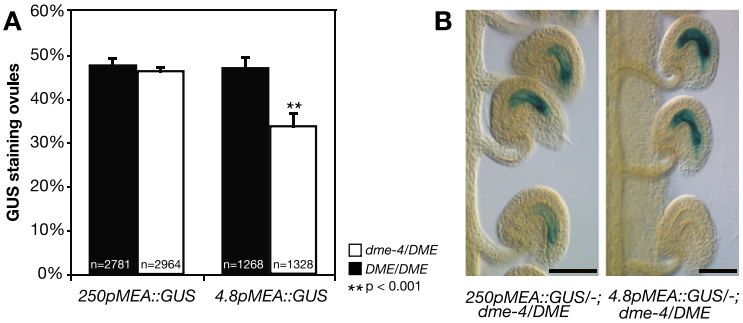

Figure 2. Maternal *MEA* activation by *DME*. (*A*) Percentage of ovules expressing the *250pMEA::GUS* and *4.8pMEA::GUS* reporter transgenes in *DME/DME* and *dme-4/DME* plants before fertilization. At least four independent *DME/DME* and four independent *dme-4/DME* segregants were analyzed for each transgene. Error bars indicate SEM. (n) Total number of ovules analyzed for each genotype; (p) level of significance relative to the difference between the two segregants (*t*-test). (*B*) Maternal *pMEA::GUS* expression of unfertilized ovules in *dme-4/DME* mutant background. Bar, 50 μm.

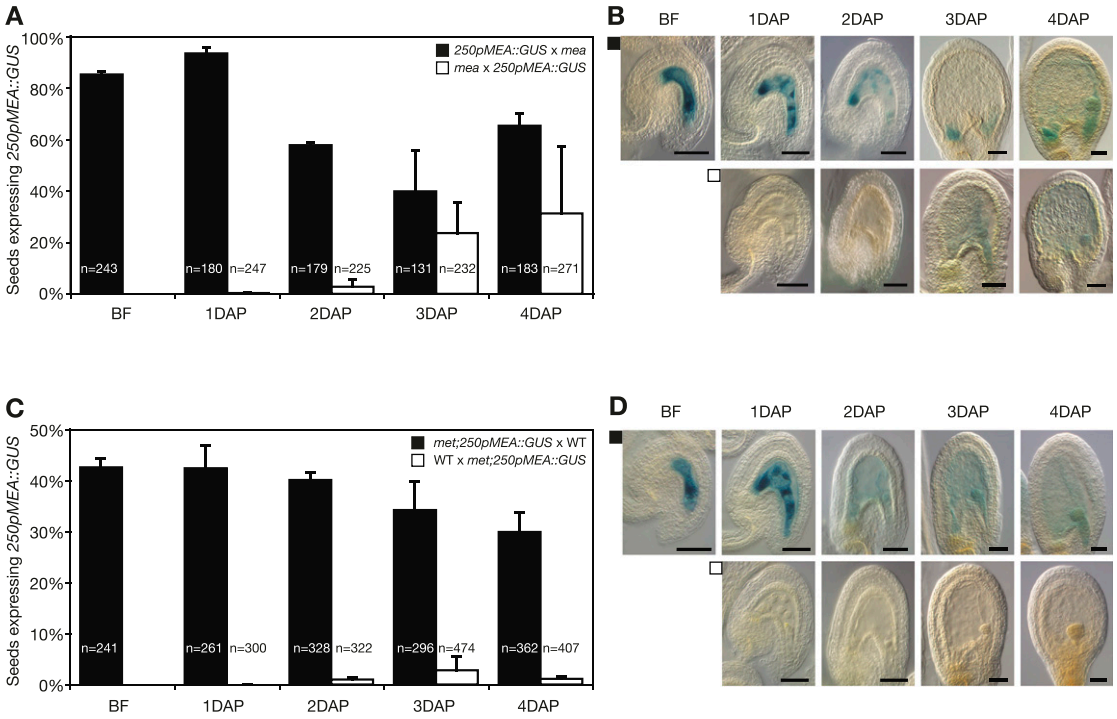

Figure 3. Expression analysis of *250pMEA::GUS* in *mea-1* and *met1-3* mutants. (*A*) Percentage of seeds expressing *250pMEA::GUS* from before fertilization (BF) until 4 DAP. Reciprocal crosses were made between plants homozygous for *250pMEA::GUS* and *mea-1*/*mea-1* (*mea*) plants. Error bars indicate SEM of two biological replicates. (n) Total number of seeds. (*B*) Maternal *250pMEA::GUS* expression was detected throughout seed development (*top* row), and weak paternal *250pMEA::GUS* expression was first detected after 3 DAP (*bottom* row). (*C*) Percentage of seeds expressing *250pMEA::GUS* from before fertilization (BF) until 4 DAP. Reciprocal crosses were made between *met1-3*/*MET* plants hemizygous for *250pMEA::GUS* (*met; 250pMEA::GUS*) and L*er* wild-type plants (WT). Error bars indicate SEM of two biological replicates. (n) Total number of seeds. (*D*) Maternal *250pMEA::GUS* expression was detected throughout seed development (*top* row), and no paternal *250pMEA::GUS* expression was detected (*bottom* row).

(Supplemental Fig. S2E), we found paternal *GUS* expression starting from 3 DAP in maternal *mea-1* mutant plants (Fig. 3A,B). The number of seeds expressing paternal *250pMEA::GUS* in the endosperm increased during development and peaked 4 DAP, with 31% of the seeds showing paternal *250pMEA::GUS* expression. Derepression during 3–4 DAP of the paternally inherited *250pMEA::GUS* transgene in maternal *mea* mutant seeds suggests that the *MEA*-ICR mediates the repressive function of the MEA–FIE complex.

MET1 is not involved in paternal transgene silencing

We found that the maternal MEA protein is required for repression of the paternal *250pMEA::GUS* transgene 3–4 DAP, which is transmitted by the pollen in a transcriptionally silent state (Gehring et al. 2006). As the paternal *MEA* allele provided by *met1* pollen showed no expression in wild-type endosperm 7 DAP, it was concluded that the methylation status of the paternal *MEA* allele is irrelevant for its transcriptional state (Gehring et al. 2006). However, derepression of the paternal *MEA* allele in a *met1* mutant background might only be visible during early seed development, when the MEA–FIE complex is not yet functionally targeting the *MEA* locus.

We tested the impact of *MET1* loss of function during male and female gametogenesis on the expression of the *250pMEA::GUS* transgene. In contrast to previous studies that did not distinguish between indirect effects of *met1-3* due to global DNA hypomethylation (Saze et al. 2003; Mathieu et al. 2007) and direct effects of *met1-3* due to *MEA* hypomethylation, we isolated *met1-3*/*MET* plants from a segregating population of wild-type plants pollinated with *met1-3*/*MET* pollen. In these plants, MET1 activity is missing only in the gametophytes, and thus pre-existing epigenetic misregulation by hypomethylation of genes other than *MEA* can be excluded. We used only *met1-3*/*MET* plants that showed full methylation at the 180-bp centromeric repeat (Martinez-Zapater et al. 1986) as an indication for wild-type methylation levels in those plants. We crossed wild-type pollen to females heterozygous for *met1-3* and hemizygous for *250pMEA::GUS* and investigated maternal GUS activity (Fig. 3C,D). We observed GUS staining in almost all prefertilization ovules and developing seeds after fertilization inheriting the reporter construct (maximum 50%) as in wild-type females (Supplemental Fig. S2C). In contrast, when we used pollen from plants heterozygous for *met1-3* and hemizygous for the *250pMEA::GUS* transgene to fertilize wild-type females, we found no (1 DAP) or only very few (2, 3, and 4 DAP) seeds with paternal

GUS activity (Fig. 3C,D). Thus, a lack of MET1 activity during female or male gametogenesis has no effect on imprinted expression of the *250pMEA::GUS* transgene.

Silencing of the endogenous paternal MEA *allele is controlled by maternal MEA*

The *MEA*-ICR in the paternally inherited *250pMEA::GUS* transgene responds to repression by maternal MEA but not by MET1. In order to correlate the control of paternal transgene silencing with the control of endogenous paternal *MEA* allele silencing, we quantified *MEA* allele-specific transcripts in *mea* mutants and combinations of *mea* mutants with *met1-3* mutants.

We first investigated the role of maternal MEA on paternal *MEA* allele silencing during early seed development. Therefore, we reciprocally crossed *MEA* wild-type plants with *mea* homozygous plants and quantified *MEA* allele-specific transcripts from 1–4 DAP (Fig. 4). Maternal transcripts in reciprocal crosses of *MEA*/*MEA* and *mea*/*mea* plants accumulated to their highest level before fertilization and decreased afterward (Fig. 4B). No paternal transcripts were detectable in a maternal *MEA* wild-type background, whereas in a maternal *mea* mutant background, paternal *MEA* allele silencing was released already at 1 DAP (Fig. 4C). Derepression of the paternal *MEA* allele continued until 4 DAP and resulted in more or less constant levels of paternal *MEA* transcripts. Remarkably, the level of derepressed paternal *MEA* transcripts in maternal *mea*/*mea* mutants represented only 19.5% (0.1563 of 0.8008) of the amount of maternal *MEA* transcripts in the maternal wild-type background (Fig. 4B,C; Supplemental Table S5). However, maternal transcription is no longer autorepressed and highly up-regulated in *mea*/*mea* mutant plants (Baroux et al. 2006), so the paternal *MEA* transcripts represented only 1.8% (0.1563 of 8.6833) of the amount of maternal *mea* transcripts in *mea*/*mea* plants (Supplemental Table S5). Thus, derepression of the paternal *MEA* allele in a maternal *mea* mutant background does not result in equivalent expression levels of the two parental alleles. The low level of derepressed paternal *MEA* expression indicates weak paternal *MEA* promoter activity, which might explain why paternal *250pMEA::GUS* expression is only detected 3–4 DAP (Fig. 3A,B). Taken together, we observed derepression of a paternally inherited *250pMEA::GUS* transgene and derepression of the endogenous paternal *MEA* allele in maternal *mea* mutants. This suggests that the *MEA*-ICR is the target of the MEA–FIE complex at the endogenous *MEA* locus.

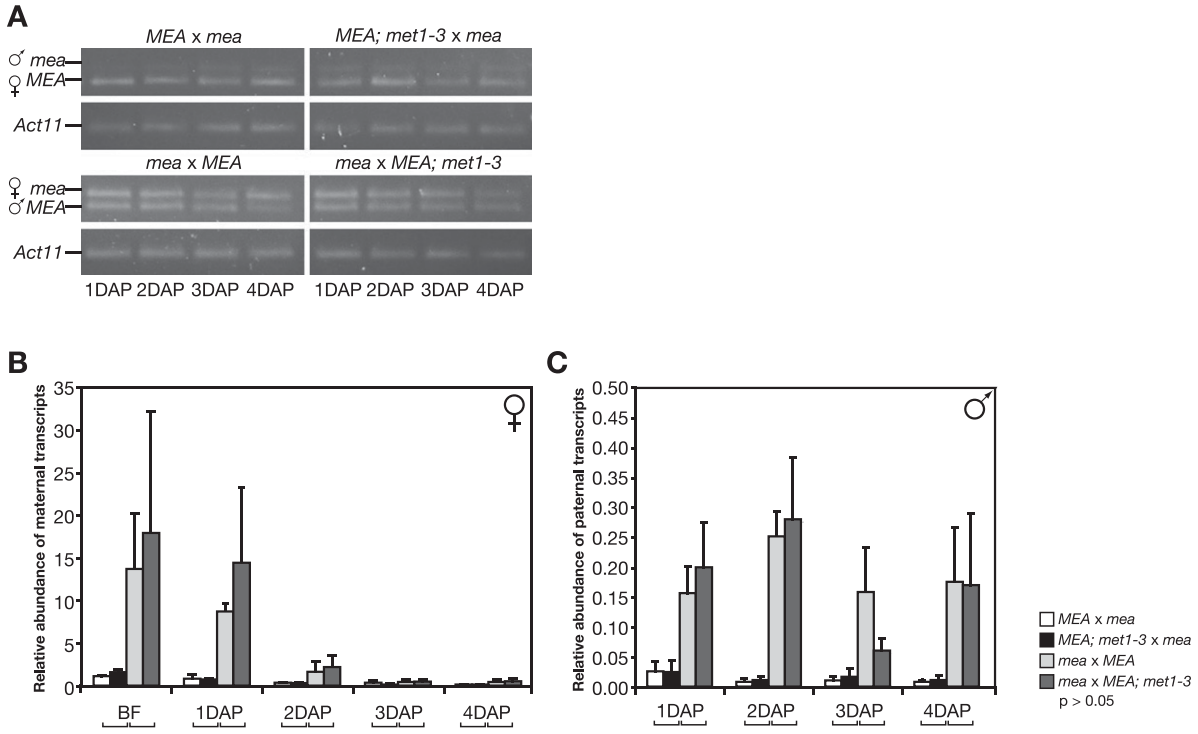

Figure 4. Quantification of *MEA* allele-specific transcription levels. (*A*) Allele-specific RT–PCR on RNA extracted from hand-pollinated siliques at 1–4 DAP. Reciprocal crosses were made between *MEA*/*MEA* (*MEA*) and *mea-2*/*mea-2* (*mea*) plants, and between *MEA*/*MEA; met1-3*/*MET* (*MEA; met1-3*) and *mea* plants. The RT–PCR products shown are the end products after 40 cycles and show qualitatively whether there is maternal and/or paternal *MEA* expression but are unsuitable to infer quantitative differences. The paternal (♂) and maternal (♀) RT–PCR products are indicated. *Actin 11* (*Act11*) was used as loading control. (*B*,*C*) Quantification of maternal (*B*) and paternal (*C*) transcripts by RT-qPCR. Transcript levels were normalized to *Act11*. No significant differences in transcript levels were found between crosses with and without *met1-3* (braces *below* the *X*-axis indicate pairwise *t*-tests). Note the different scales for maternal and paternal transcripts. Error bars indicate SEM of three biological replicates.

Since a lack of maternal MEA–FIE PcG activity only leads to very weak derepression of the endogenous paternal *MEA* allele, we wondered whether DNA methylation might be involved in keeping it largely silenced. Thus, we asked again whether MET1 has any residual role in paternal *MEA* allele silencing and crossed *mea*/*mea* mother plants with either wild-type or *met1-3* mutant pollen and analyzed allele-specific *MEA* expression levels (Fig. 4A,C). In *mea*/*mea* mutant mothers, the paternal *MEA* allele was derepressed when transmitted by both wild-type and *met1-3* pollen, with no significant change in the level of derepression (Fig. 4C). This shows that MEA, presumably as part of the maternal MEA–FIE complex, represses the paternal *MEA* allele independent of its methylation status maintained by MET1 during male gametogenesis. Thus, even after removal of both known repressing factors, the maternal MEA–FIE complex and MET1, the paternal *MEA* allele is still expressed at extremely low levels compared with the maternal *MEA* allele; in other words, it is still imprinted. Furthermore, we detected no paternal *MEA* transcripts in the reciprocal cross when *mea* homozygous mutant pollen was crossed to *met1-3* heterozygous females. We conclude that paternal MET1 during male gametogenesis and maternal MET1 during early seed development are not required for *MEA* paternal silencing and thus play no significant role in imprinting at the *MEA* locus.

The MEA-*ICR is unmethylated*

Our comparative analysis of *MEA* transgene and endogene regulation revealed that the *MEA*-ICR is not targeted by DME and MET1. Thus, contrary to what was previously suggested (Gehring et al. 2006; Jullien et al. 2006a), *MEA* imprinting regulation is not primarily controlled by differential DNA methylation. Therefore, we speculated that there is either no DNA methylation at all at the *MEA*-ICR or no differential DNA methylation between active and silent *MEA* alleles.

We analyzed *MEA* promoter methylation in isolated central cells and sperm cells as well as in isolated two-cell stage embryos where the maternal *MEA* allele is expressed (Vielle-Calzada et al. 1999; Spillane et al. 2007). In parallel, we monitored *FWA* promoter methylation, which exhibits imprinting control through a differentially methylated SINE-related element in its promoter (Kinoshita et al. 2004, 2007). In sperm cells, we found high levels of *FWA* promoter methylation in the CG context, consistent with previously reported methylation levels in pollen (Fig. 5E,G; Kinoshita et al. 2004). Surprisingly, we found only a small reduction of CG methylation in the central cell at the *FWA* locus, suggesting that DNA methylation is fully removed after fertilization only (Fig. 5E,F). Contrary to this, we detected almost no methylation in the 250-bp *MEA* promoter from sperm cells and central cells in any sequence context (Fig. 5A–C). In addition, we analyzed methylation in two-cell stage embryos early after fertilization. We detected high methylation levels of *FWA* in the CG contexts in the embryo (Fig. 5E,H), consistent with MET1-dependent silencing of parental *FWA* alleles. However, we found no methylation in the 250-bp *MEA* promoter in the embryo, where the maternal *MEA* allele is expressed (Fig. 5A,D).

In summary, MET1-dependent *FWA* silencing in sperm cells, central cells, and the embryo correlates with DNA methylation in the SINE-related repeat region of its promoter. However, the *MEA*-ICR in the 250-bp *MEA* promoter carries no DNA methylation in any reproductive cell. This confirms our finding that DME is not targeted to the *MEA*-ICR for maternal *MEA* allele activation and that MET1 is not involved in paternal *MEA* allele silencing. Thus, *MEA* is regulated differently from *FIE* and *FWA*, and presently unknown factors, together with the MEA–FIE complex, must be responsible for the imprinted expression of *MEA*.

Discussion

The MEA-*ICR maps to a 200-bp region and displays no differential DNA methylation*

In plants, the primary DNA sequences responsible for genomic imprinting remained elusive. Studies involving transgenes to identify the *cis*-determinants for imprinted expression in *Arabidopsis* and maize indicated that plants ICRs are located close to the imprinted loci (Luo et al. 2000; Kinoshita et al. 2004; Gehring et al. 2006; Gutierrez-Marcos et al. 2006; Makarevich et al. 2008). We identified the 200-bp upstream region adjacent to the *MEA* translational start site as the minimal sequence necessary to confer *cis*-activation and imprinted expression of a *GUS* transgene. The proximity of the *MEA*-ICR and the *MEA* locus is in contrast to mammalian ICRs, which can be located >100 kb distal from the imprinted loci (Ferguson-Smith and Surani 2001).

Mammalian ICRs are typically a few kilobases in length and exhibit parental allele-specific DNA methylation (Bartolomei 2009). However, the *MEA*-ICR maps to a 200-bp fragment and is essentially unmethylated, excluding DNA methylation as the epigenetic mark distinguishing maternal and paternal *MEA* alleles. This is in contrast to the *cis*-elements involved in imprinting at the *FWA* and *PHE1* loci. Maternal-specific expression of *FWA* in the endosperm is due to differential methylation of a SINE-related element located in the *FWA* promoter (Kinoshita et al. 2007). Yet our analysis of DNA methylation in gametes shows that differential methylation at *FWA* is only established after fertilization. This suggests that the primary germline imprint at the *FWA* locus is not the DNA methylation mark itself. Imprinting of *PHE1* results in preferential paternal expression in the endosperm and correlates with differential methylation of tandemly repeated motifs located 3 kb downstream from the *PHE1* gene (Makarevich et al. 2008). Furthermore, differential DNA methylation between the parental alleles has been described for the maize imprinted genes *ZmFie1* and *ZmFie2* (Gutierrez-Marcos et al. 2006). Interestingly, *ZmFie2* is unmethylated in both central cells and sperm cells prior to fertilization, and the differential methylation pattern is only established after fertilization,

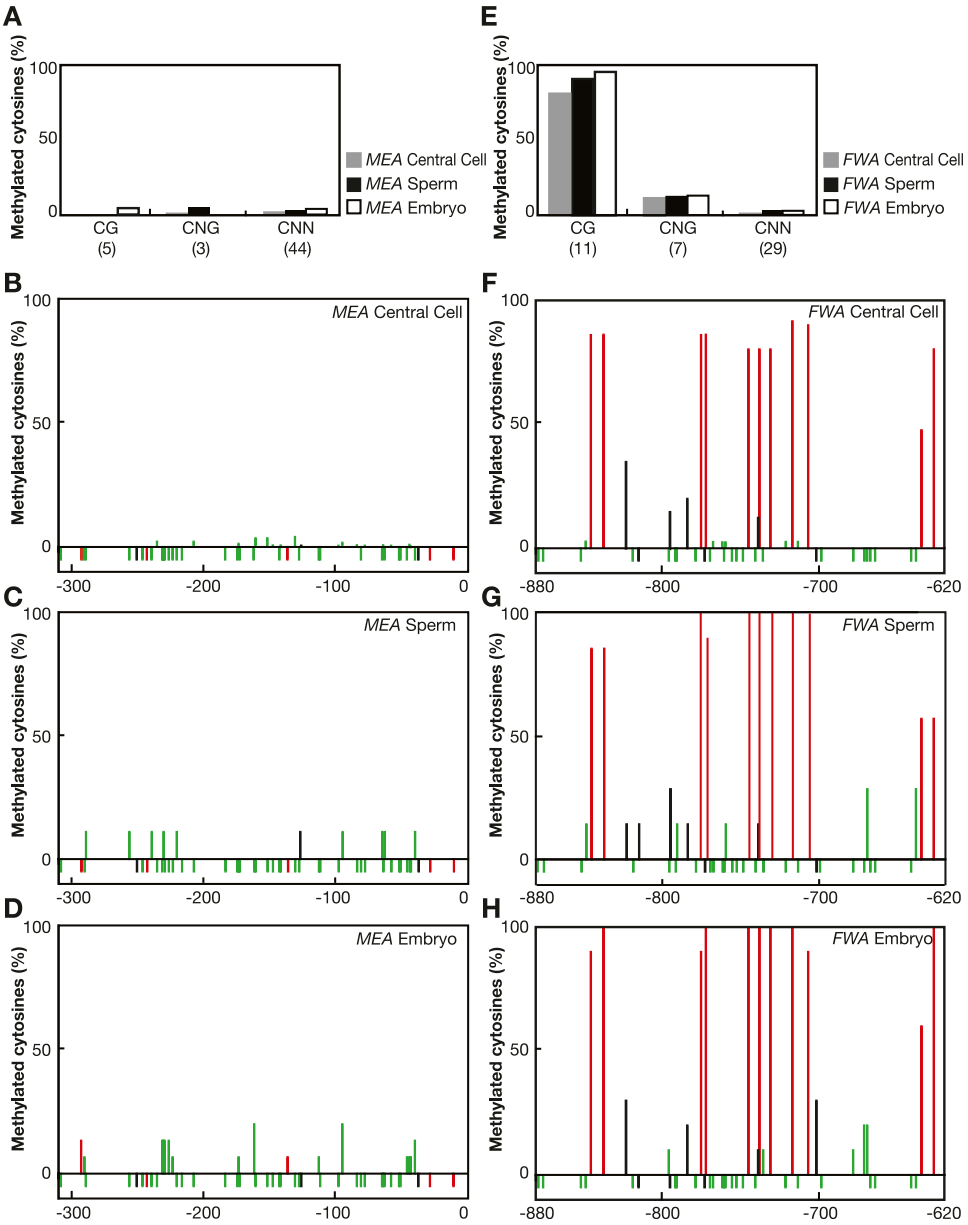

Figure 5. Promoter methylation of *MEA* and *FWA*. (*A*,*E*) Percentage of cytosine methylation at CG, CNG, and CNN sites in the *MEA* promoter (*A*) and the *FWA* promoter (*E*). DNA was isolated from central cells, sperm cells, and two-cell stage embryos; bisulfite-treated; sequenced; and analyzed. Numbers in brackets indicate the number of sites present in the investigated promoter region. (*B–D*,*F–H*) Percentage of cytosine methylation at each position is indicated with a red (CG), black (CNG), or green (CNN) bar. Unmethylated cytosines are shown *below* the 0% line. Numbers are relative to the translational start site and indicate the investigated promoter region.

also indicating that the primary germline imprint is not a DNA methylation mark. In addition, several of the potentially imprinted genes recently identified by transcriptome profiling are unaffected by mutations in one or even all of the known imprinting factors (i.e., *DME*, *MET1*, and *FIE*) (Hsieh et al. 2011; Wolff et al. 2011), suggesting additional, yet-undiscovered imprinting regulators.

MEA is an imprinted gene that is not controlled by differential DNA methylation at the ICR. A related situation may occur in the mouse Prader-Willi/Angelman region showing a complex imprinting control involving several *cis*-acting elements, one of which is not differentially methylated but is required to establish parental imprints at other sites (Kaufman et al. 2009). Moreover, it was recently shown that in macaques, some ICRs that acquire a germline DNA methylation imprint in mice are not methylated in the germline and acquire a differential methylation mark only post-fertilization (A Ferguson-Smith, pers. comm.). Thus, primary imprints that do not involve germline DNA methylation appear to exist in

both plants and mammals. Future studies will show whether common regulatory mechanism indeed exist between nonmethylated ICRs in mammals and plants.

Imprinting control at the MEA*-ICR is independent of DME and MET1*

Maternal allele expression of *MEA* and other maternally expressed imprinted genes depends on the removal of MET1-dependent DNA methylation (Choi et al. 2002; Kinoshita et al. 2004; Jullien et al. 2006b). Consistent with the lack of significant DNA methylation at the *MEA*-ICR, the imprinted *250pMEA::GUS* transgene is maternally activated independent of DME, suggesting that DME is only required in the endogenous context, probably targeting a region different from the *MEA*-ICR. Although involved in imprinting, DNA methylation in flowering plants primarily silences transposons and repeat elements (Henderson and Jacobsen 2007). Thus, a 590-bp *AtREP2* transposon element that is located −4 kb upstream of the *MEA* start codon represents a likely DME target. Indeed, the previously described *4.2pMEA::GUS* transgene containing 450 bp of the *AtREP2* is fully dependent on DME for activation (Choi et al. 2002), whereas the *4.8pMEA::GUS* transgene containing 3.8 kb of *MEA* upstream sequence with 100 bp of the *AtREP2* is only partially dependent on DME (this study). Therefore, we hypothesize that DME is only indirectly involved in the activation of endogenous maternal *MEA* transcription by demethylation of the *AtREP2*.

Based on our results, we propose a new model of *MEA* imprinting regulation (Fig. 6). The methylated *AtREP2* would interact with an unidentified region of the *MEA* locus to establish a silent higher-order chromatin structure; e.g., a repressive chromatin loop. This prevents the *MEA* promoter from being accessed by an unknown transcriptional activator binding the *MEA*-ICR. Demethylation of *AtREP2* by DME in the central cell resolves the repressive chromatin loop and allows the transcriptional activator to access the *MEA*-ICR. The repressive chromatin loop is not resolved in the male gametophyte, where *DME* is not expressed, resulting in exclusive maternal *MEA* allele expression. Since the paternal *MEA* allele is not fully activated if both known repressing activities, MET1 and the MEA–FIE complex, are removed, additional paternal repressors involved in imprinting control have to be postulated, possibly including a PcG complex with a histone methyltransferase other than MEA.

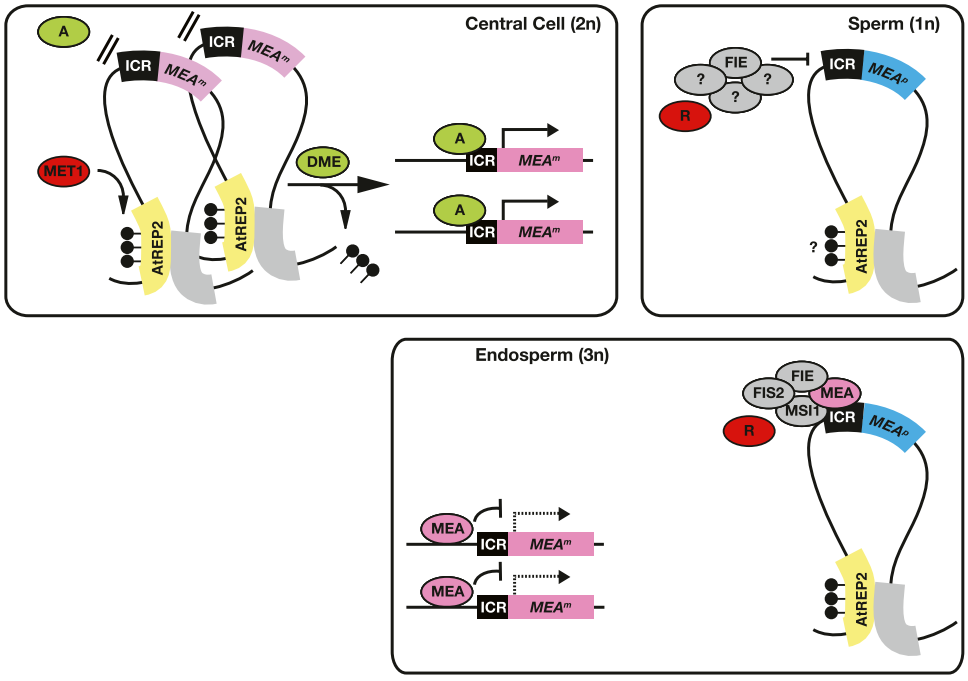

Figure 6. Model of *MEA* imprinting control through a higher-order chromatin structure. Methylation at *AtREP2* is maintained by MET1 in central cells and sperm cells. *AtREP2* might interact with another region, thereby forming a repressive chromatin loop preventing the *MEA* locus from being accessed by a transcriptional activator (A). Specific expression of *DME* in the central cell removes methylation and resolves the repressive chromatin loop. This allows the transcriptional activator (A) to access the *MEA*-ICR. As a consequence, in the endosperm, the two maternal *MEA* alleles (MEA^m) are expressed. Paternal *MEA* allele (MEA^p) silencing is maintained by a proposed PcG complex containing FIE during male gametogenesis (Jullien et al. 2006a). After fertilization, MEA^p is repressed partially by the maternal MEA–FIE complex and another paternal repressor (R). Since parental alleles in the endosperm are differentially targeted by *trans*-acting factors, they must have been marked in the germline, as illustrated by the purple and blue color of the maternal and paternal allele, respectively. The nature of this mark is unknown. The model explicitly shows MEA^m activation in the central cell for imprinted expression in the endosperm; however, the same model is proposed for the egg cell and embryo. (Lollipops) DNA methylation; (dashed line) autorepressed MEA^m transcription.

In mammals, chromosome conformation capture experiments revealed that chromosome looping is involved in imprinting control (Lopes et al. 2003; Kurukuti et al. 2006; Yoon et al. 2007; Engel et al. 2008). More specifically, interactions of differentially methylated regions (DMRs) at the mouse H19/Igf2 locus were shown to partition maternal and paternal chromatin into distinct loops, generating an epigenetic switch to control allele-specific expression (Murrell et al. 2004). Our findings raise the possibility that *MEA* imprinting control might depend on a similar mechanism involving higher-order chromatin structure controlled by DME and MET1.

This hypothesis is consistent with recent reports that *DME* is involved in genome-wide demethylation of the maternal genome in the endosperm, especially of transposons and repeat elements (Gehring et al. 2009; Hsieh et al. 2009). Intriguingly, all characterized imprinted genes in plants are hypomethylated on the maternal allele regardless of which allele is expressed. This suggests that DME-dependent demethylation in the endosperm is not specifically targeting imprinted genes, but rather is a nearly universal process that reshapes DNA methylation of the entire maternal genome in the endosperm.

The imprinting factors required for paternal MEA *silencing remain unknown*

Two epigenetic silencing marks were found at specific sites of the *MEA* locus: DNA methylation and histone H3K27 di- and trimethylation (H3K27me) (Xiao et al. 2003; Gehring et al. 2006; Jullien et al. 2006a). We report that lack of MET1 during male gametogenesis does not derepress the paternal *MEA* allele 1–4 DAP. This complements previous studies with *met1* mutant pollen that showed no paternal *MEA* allele expression 7–9 DAP (Gehring et al. 2006).

Whereas DNA methylation is irrelevant for paternal *MEA* allele silencing, PcG-mediated histone methylation is necessary for paternal *MEA* allele silencing (Gehring et al. 2006; Jullien et al. 2006a). Maternal MEA is involved in deposition of repressive H3K27me at the paternal *MEA* allele close to the translational start site (Gehring et al. 2006). We found derepression of a paternally inherited *250pMEA::GUS* transgene in the maternal *mea* mutant background, suggesting that the *MEA*-ICR in the 250-bp *MEA* promoter is targeted by the maternal MEA–FIE complex. However, it is unclear how the MEA–FIE complex gains access to the silent chromatin loop of the paternal allele to maintain silencing after fertilization. Possibly, the repressive machinery, including the MEA–FIE complex and other proposed repressors, has access to *cis*-regulatory elements in repressive chromatin loops, whereas the activating machinery is efficiently prevented from binding to the *MEA*-ICR.

We found derepression of the paternal *MEA* allele in the maternal *mea* mutant background already at 1 DAP. This contradicts recent findings of delayed paternal derepression, which were explained by the need for passive loss of repressive H3K27me on the paternal *MEA* allele (Jullien et al. 2006a). Surprisingly, derepressed paternal *MEA* transcripts in maternal *mea* mutant plants represent only 14% of maternal *MEA* transcripts in maternal wild-type plants. This resembles the observed residual transcriptional activity of the silent maternal *PHE1* allele (Köhler et al. 2005). Similarly, in mice, paternal alleles of several imprinted genes in the IC2-imprinted domain are not completely silent (Lewis et al. 2004). Even though the silent paternal *MEA* allele is derepressed in *mea* mutant plants, parental transcript levels are clearly not equivalent and still show parent-of-origin-dependent differences. Assuming equivalent parental expression levels in the background of compromised imprinting, the main components involved in paternal *MEA* allele silencing remain to be identified because the paternal *MEA* allele is still imprinted when MET1 and the MEA–FIE complex are missing. As the *MEA*-ICR confers paternal *MEA* silencing beyond the native genomic context, loop formation is not sufficient to explain paternal *MEA* silencing. Thus, another unknown repressor binding to the *MEA*-ICR, along with the proposed PcG complex (Jullien et al. 2006a), may be required for paternal *MEA* repression (Fig. 6).

In summary, our promoter dissection identified the *MEA*-ICR in the 200-bp *MEA* upstream sequence. The *MEA*-ICR carries no significant methylation in sperm cells, central cells, and two-cell stage embryos, which to our knowledge is the first example of an ICR without differential DNA methylation. DME, the key factor necessary for specific activation of maternally expressed imprinted genes in *Arabidopsis*, is dispensable for activation of maternal *MEA* allele transcription. Instead, DME and MET1 may be involved in the regulation of a higher-order chromatin structure at the *MEA* locus, thereby only indirectly controlling the specific marking and activation of the maternal *MEA* allele by unknown factors. However, a repressive chromatin structure at the paternal *MEA* locus alone cannot explain paternal *MEA* silencing, which is mediated through the *MEA*-ICR beyond the native genomic context by still unknown *MEA* imprinting factors.

Material and methods

Plant material

The L*er* accession was used as the wild type. The mutant alleles used were *mea-1*, *mea-2* (L*er*) (Grossniklaus et al. 1998), *dme-4* (C24) (Guitton et al. 2004), and *met1-3* (Col) (Saze et al. 2003). The *4.8pMEA::GUS* transgenic line was described before (Spillane et al. 2004). The *dme-4* (C24) and the *met1-3* (Col-0) mutants were introgressed into the L*er* background by crossing them at least five times as pollen parents. For genotyping assays, methylation status evaluation, and growth conditions, see the Supplemental Material.

Generation of pMEA::MEA *and* pMEA::GUS *constructs*

All *pMEA::MEA* constructs were cloned into pCAMBIA3300 containing the corresponding *MEA* promoter sequence and the entire *MEA* ORF amplified from genomic L*er* DNA. All *pMEA::GUS* constructs contain the corresponding *MEA* promoter sequence amplified from genomic L*er* DNA and were cloned in-frame to the *GUS* reporter gene in pCAMBIA 1381Z.

Promoter deletions were done using different primer pairs amplifying differently sized amplicons and were subsequently cloned in the above-mentioned vectors. For a detailed cloning procedure, see the Supplemental Material.

Microscopy and GUS staining

Histochemical analysis of GUS reporter gene expression was essentially done as described in Baroux et al. 2006. Microscopic inspection was carried out under differential contrast (DIC) optics using a Leica DMR microscope (Leica Microsystems). A detailed description can be found in the Supplemental Material.

RT–PCR analyses

Reverse transcription was performed as previously published (Baroux et al. 2006) on 20 gynoecia before fertilization or on 10–15 siliques at 1–4 DAP, depending on the stage indicated in the corresponding figure. In all experiments, transcript levels were normalized to the level of *ACTIN11* (Huang et al. 1997). For detailed protocol and primers used, see Supplemental Material.

Bisulfite DNA sequencing of isolated reproductive cells

Central cells were isolated using laser capture microscopy, sperm cells were isolated using a Percoll density gradient (M Schauer and U Grossniklaus, unpubl.), and embryos were isolated as previously described (Autran et al. 2011). DNA isolation and bisulfite conversion were essentially performed as described in the Epigenetics Protocols Database "Bisulphite sequencing of small DNA/cell samples" (PROT35; http://www.epigenome-noe.net/research tools/protocols.php). Subsequently, regions of interest (250-bp *MEA* promoter and SINE-related tandem repeat in the *FWA* promoter) were amplified. Purified bisulfite PCR products were cloned into the pGEM-T vector (Promega) and several independent clones were sequenced (for sperm cell and embryo sample), or purified PCR products were directly sequenced with the 454 sequencer according to the standard protocol (central cell samples).

All sequences were analyzed with the BiQ Analyzer software (Bock et al. 2005) for quality control and removal of identical clones in a standardized manner. For a more detailed description, see the Supplemental Material.

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