Mechanism of *PHERES1* imprinting in *Arabidopsis*

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Summary

Genomic imprinting is a phenomenon where only one of the two alleles of a gene is expressed – either the maternally or the paternally inherited allele. Imprinting of the plant gene *PHERES1* requires the function of the FERTILIZATION INDEPENDENT SEED (FIS) Polycomb group (PcG) complex for repression of the maternal *PHERES1* allele. In this study we investigated the mechanism of *PHERES1* imprinting and found that PcG silencing is necessary but not sufficient for imprinting establishment of *PHERES1*. We provide evidence that silencing of the maternal *PHERES1* allele depends on a distantly located region downstream of the *PHERES1* locus. This region needs to be methylated to ensure *PHERES1* expression but must not be methylated for *PHERES1* repression. This mechanism is analogous to the regulation of several imprinted genes in mammals, suggesting the employment of similar evolutionary mechanisms for the regulation of imprinted genes in mammals and flowering plants.

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Key words: *Arabidopsis*, Epigenetics, *FERTILIZATION INDEPENDENT SEED* genes, DNA methylation, Polycomb group proteins

Introduction

A subset of genes in mammals and flowering plants is only expressed from one of the two homologous chromosomes, depending on whether they are maternally or paternally inherited. This process is called genomic imprinting and often affects genes with essential functions for normal development (Feil and Berger, 2007). In mammals, imprinted genes regulate placental development and fetal growth, and several human diseases are linked to mutations in imprinted genes (Reik, 2007). Mechanisms to distinguish maternal and paternal alleles have been extensively investigated in mammals. Most mammalian imprinted genes are clustered in the genome and are regulated by differentially methylated imprinting-control regions (ICRs) (Edwards and Ferguson-Smith, 2007). Different DNA methylation marks are applied during germ-line formation by de novo methyltransferases and are maintained in somatic tissues by maintenance methyltransferases. ICRs are also marked by different histone modifications and can either act as insulators preventing promoter-enhancer interactions or give rise to the formation of non-coding RNAs that attract chromatin-modifying complexes (Delaval and Feil, 2004). Some imprinted genes are regulated by Polycomb group (PcG) complexes that methylate histones and establish repressive chromatin domains (Delaval and Feil, 2004).

In flowering plants, imprinting has only been detected in the endosperm, a terminal tissue that develops after fertilization of the central cell. In dicot species like *Arabidopsis*, the endosperm nourishes the embryo during its growth phase and is almost completely consumed during embryo development (Berger, 2003). Thus far, only four imprinted genes have been identified in Arabidopsis. Three of them [*MEDEA* (*MEA*), *FWA* and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*)] are maternally expressed and paternally silenced (Vielle-Calzada et al., 1999; Kinoshita et al., 2004; Jullien et al., 2006b). The same paternally imprinted expression pattern applies to all imprinted genes that have been identified in maize (Scott and Spielman, 2006). Paternal imprinting of *MEA* requires activity of the evolutionary conserved FIS-PcG complex, with MEA itself being a subunit of this complex. The FIS complex mediates trimethylation of histone H3 at the lysine residue at position 27 (H3K27me3) of the paternal *MEA* allele causing its repression (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006a). Activation of the maternal *MEA* allele in the female gametophyte requires the 5-methylcytosine excising activity of the DNA glycosylase DEMETER (DME). DME acts antagonistically to the maintenance methyltransferase MET1 that methylates *MEA* in the promoter and 3′ regions of the gene (Xiao et al., 2003). DME activity is also necessary to activate expression of the paternally imprinted genes *FWA* and *FIS2*. Because *MEA*, *FWA* and *FIS2* are only demethylated in the terminally differentiating endosperm, they remain heritably methylated throughout the life cycle of the plant (Xiao et al., 2003; Kinoshita et al., 2004; Jullien et al., 2006b).

The promoter region of *FWA* and the 3′ region of *MEA* contain tandem-repeat sequences that recruit de novo DNA methylation by the RNA-dependent DNA silencing pathway (Chan et al., 2006b). Whereas *FWA* tandem repeats are necessary to establish *FWA* silencing (Chan et al., 2006b), silencing of the paternal *MEA* allele is likely to be independent of the repeat sequences (Spillane et al., 2004).

The type I MADS-box gene *PHERES1* (*PHE1*) is the only plant gene known to be paternally expressed and maternally silenced. Maternal *PHE1* silencing is caused by the repressing activity of the FIS complex (Köhler et al., 2005). The FIS complex is active in the female gametophyte and in the endosperm, prevents precocious *PHE1* expression before fertilization and restricts *PHE1* expression to the chalazal domain of the endosperm after fertilization. The FIS complex is directly associated with the *PHE1* locus and FIS repressive activity is correlated with H3K27me3 modification at *PHE1* (Köhler et al., 2003; Makarevich et al., 2006). Upregulation

of *PHE1* in *mea* mutants is in part responsible for the *mea* mutant phenotype that can be alleviated by reducing *PHE1* expression (Köhler et al., 2003).

In this study, we asked whether FIS-mediated repression is sufficient for *PHE1* imprinting or whether additional mechanisms are involved to repress the maternal *PHE1* allele. Our data clearly show that imprinting is not a direct consequence of FIS-mediated repression but necessitates the presence of additional elements. We identified elements within the *PHE1* 3′ region that are necessary for *PHE1* imprinting and predict a model that explains how the FIS complex – together with the identified region – confers stable silencing of the maternal *PHE1* allele.

Results

The maternal *PHE1* allele is not reactivated in mutants that are defective in DNA methylation

We asked the question whether the FIS complex is sufficient to suppress the maternal *PHE1* allele, or whether additional mechanisms cooperate with the FIS complex to silence the maternally derived *PHE1* allele. Parental allele-specific DNA methylation has been found at most imprinted mammalian gene clusters and imprinted plant genes that have been examined (Köhler and Makarevich, 2006; Edwards and Ferguson-Smith, 2007). Therefore, we tested whether imprinting of *PHE1* is also regulated by DNA methylation. Mutations in the maintenance *MEHYLTRANSFERASE 1* (*MET1*) gene cause a drastic reduction of symmetric CG methylation (Kankel et al., 2003), whereas mutations in the *CHROMOMETHYLASE 3* (*CMT3*) gene and the de novo methyltransferases *DRM1* and *DRM2* affect CNG and asymmetric methylation, respectively (Cao and Jacobsen, 2002). We pollinated *met1*, *cmt3* and *drm1/drm2* double mutants with pollen of C24 wild-type plants and analyzed allele-specific expression of *PHE1*. As shown in Fig. 1A and supplementary material Fig. S1A and Fig. S3, in none of the mutants a reactivation of the maternal *PHE1* allele was detectable and the paternal *PHE1* allele remained the predominantly expressed allele. In conclusion, DNA methylation is not responsible for repression of the maternal *PHE1* allele.

Lack of DNA methylation causes reduced expression of the paternal *PHE1* allele

In mammals, DNA demethylation is responsible for silencing of a large number of imprinted protein-coding genes (Sleutels and Barlow, 2002). Previously we observed strongly reduced *PHE1* expression levels in the *decrease in DNA methylation 1* (*ddm1*) mutant, which is impaired in the maintenance of DNA methylation (Köhler et al., 2003). This prompted us to test whether DNA methylation is required for expression of the paternal *PHE1* allele. We did not detect substantial *PHE1* expression in pollen (data not shown); however, fertilization of wild-type plants with hypomethylated pollen of *met1* mutants caused much lower *PHE1* transcript levels in developing seeds than fertilization with wild-type pollen (Fig. 2A), indicating reduced activity of the paternal *PHE1* allele. This suggests that DNA methylation is important to maintain high levels of paternal *PHE1* expression, but is not necessary to repress the maternal *PHE1* allele. When we tested allele-specific *PHE1* expression in seeds developing after pollination with *met1* pollen, we clearly detected reduced expression of the paternal *PHE1* allele (Fig. 2B), which supports our conclusion that DNA methylation is required for paternal *PHE1* expression. However, we also detected expression of the maternal *PHE1* allele in seeds that inherited a hypomethylated paternal *PHE1* allele (Fig. 2B and supplementary material Fig. S1B), suggesting that relieve of repression of the maternal *PHE1* allele depends on additional not-yet-known factors. Because DNA methylation appears to be important for *PHE1* regulation, we asked the question which regions

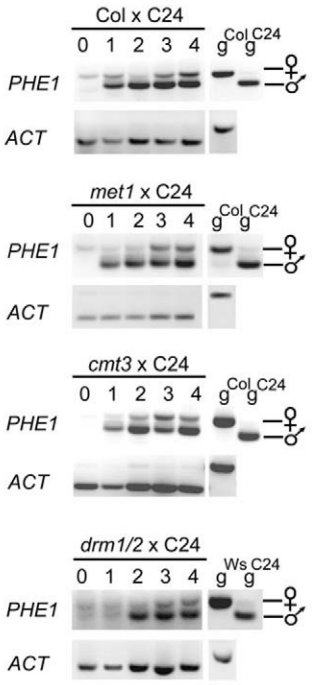

Fig. 1. The maternal *PHE1* allele is not reactivated in DNA-methylation-defective mutants. Allelic expression analysis of *PHE1* in wild-type and *met1*, *cmt3* and *drm1/drm2* mutant plants after crosses of wild-type and mutant plants with the C24 accession. Analysis was performed before fertilization (0 DAP) and at indicated days after pollination (DAP). g, genomic DNA.

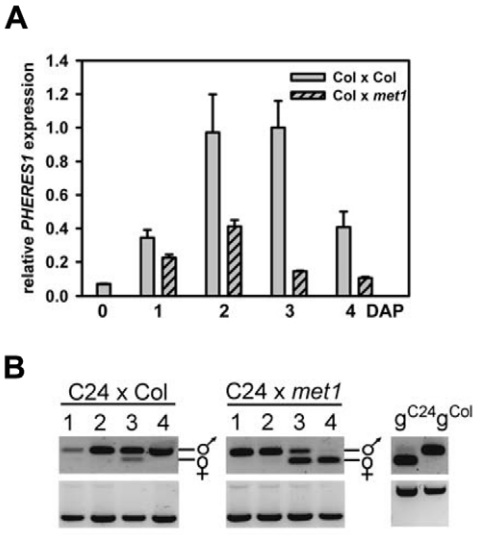

Fig. 2. DNA methylation is important for paternal *PHE1* expression. (A) Relative *PHE1* mRNA levels in wild-type flowers before fertilization (0 DAP) and siliques after pollination with wild-type or *met*-mutant pollen harvested at different days after pollination (DAP). Because the maternal *PHE1* allele is silent, the detected *PHE1* expression represents mostly the activity of the paternal *PHE1* allele. (B) Allelic expression analysis of *PHE1* after pollination of C24 plants with wild-type or *met1*-mutant pollen. Analysis was performed at DAP1 to DAP4.

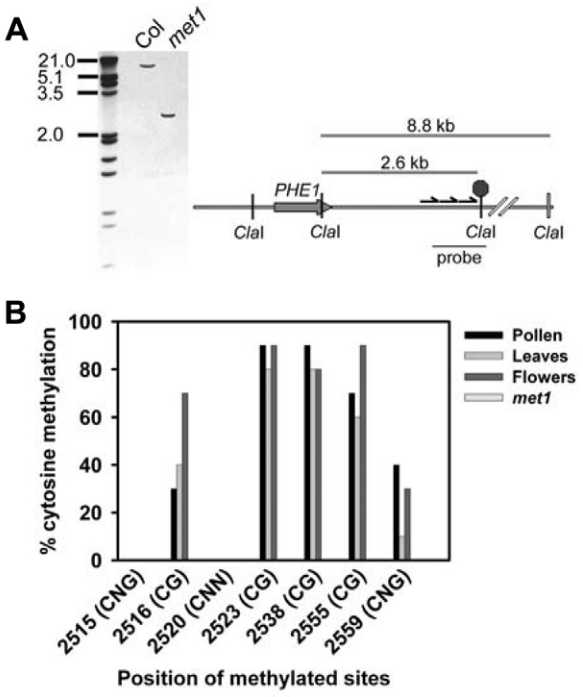

Fig. 3. The 3′ region of *PHE1* contains DNA-methylation marks. (A, left) Southern blot of DNA from wild-type and *met1*-mutant plants after digestion with the methylation-sensitive restriction enzyme *Cla*I . (Right) Overview of the *PHE1* locus with large and small arrows corresponding to the *PHE1* coding region and repeats, respectively. The expected fragment sizes and the region covered by the probe are indicated. The methylated *Cla*I site is indicated by a filled circle, the repeat regions by arrows. (B) Cytosine methylation profile of the *PHE1* downstream region in different tissues analyzed by bisulfite sequencing. Cytosine positions relative to the translational stop codon and sequence contexts are indicated on the *x*-axis. The *Cla*I site corresponds to position 2538. The methylation status in the *met1* mutant was analyzed in flowers.

of the *PHE1* locus contain DNA methylation. Using bisulfite sequencing we did not detect any DNA methylation within the promoter and coding regions of *PHE1*. Therefore, we extended our analysis to regions downstream of the *PHE1* locus. We performed Southern blot analysis using the restriction enzyme *Cla*I, which is blocked by symmetric methylation at its target recognition site. Using this assay we detected a methylated site 2538 bps downstream of the *PHE1* stop codon (Fig. 3A). No annotated genes are located in this region. Using Southern blot analysis and bisulfite sequencing we detected no substantial methylation at this site in the *met1* mutant (Fig. 3A,B). Thus, reduced methylation at this site in *met1* correlates with reduced *PHE1* expression. Interestingly, this methylated *Cla*I site is located in close proximity to a tandem triple repeat, each repeat being 54 bps long. Bisulfite sequencing of the region surrounding the *Cla*I site revealed twelve consecutive methylated cysteine residues in a symmetric CG context and two methylated CNG sites (supplementary material Fig. S3). We performed tissue-specific analysis of DNA methylation in pollen, flowers and leaves. Consistent with our findings that in *met1* mutants the paternal allele is less expressed, we detected high levels of DNA methylation in pollen at all sites tested (Fig. 3B). However, we also detected substantial DNA methylation in sporophytic tissues where only weak *PHE1* expression is detectable, suggesting that DNA methylation alone is not sufficient to confer active *PHE1* expression.

Disruption of the *PHE1* 3′ regions causes activation of the maternal *PHE1* allele

Our results suggest that DNA methylation in the identified *PHE1* 3′ region is important for *PHE1* imprinting. In order to test this hypothesis we addressed the question whether disruption of the *PHE1* 3′ region disrupts *PHE1* imprinting. We identified a T-DNA mutant containing a 4 kb T-DNA insertion 441 bps downstream of the *PHE1* stop codon (referred to as *phe1-3*) (Fig. 4A). Using this line, we could test whether an insertion within the 3′ region disrupts *PHE1* imprinting. We tested allele-specific expression of *PHE1* in *phe1-3* by performing reciprocal crosses of *phe1-3* with C24 plants. Whereas expression of the paternal *PHE1* allele was not affected in *phe1-3* (Fig. 4B, upper panel and supplementary material Fig. S1C), we observed a drastic effect on the expression of the maternal allele when *phe1-3* was used as the maternal parent (Fig. 4B lower panel and supplementary material Fig. S1C). In *phe1-3* mutants, *PHE1* was not maternally silenced but, in contrast to wild-type, which has only a weak expression of the maternal *PHE1* allele, this allele was strongly expressed (Fig. 4B, lower panel). Surprisingly, we detected a decrease in the expression of the paternal *PHE1* allele when *phe1-3* was used as the maternal parent (cross *phe1-3* × C24; Fig. 4B and supplementary material Fig. S1C). One possible explanation for this phenomenon might be the triploid nature of the endosperm consisting of two maternal versus one paternal genome copies. Therefore, if the two maternal *PHE1* alleles become reactivated, they might outcompete expression of the single paternal *PHE1* allele. We also considered the possibility that strong expression of the maternally derived *PHE1* allele in *phe1-3* is caused by de-repression of *PHE1* in maternal sporophytic tissues of *phe1-3* plants. Therefore, we tested expression of *PHE1* in *phe1-3* leaves. However, as shown in Fig. 4C, *PHE1* remains as weakly expressed in *phe1-3* leaves as in wild-type leaves. We further considered the possibility that transcripts derived from the T-DNA can influence *PHE1* expression. To test this possibility, we performed northern blot analysis using probes flanking the insertion site. However, we did not obtain any expression signal with either of the probes, but detected a clear expression signal for the *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) selection gene that is located within the T-DNA (Fig. 4D). Finally, we tested *PHE1* imprinting in two additional transgenic lines containing a 4 kb T-DNA or a 6.6 kb *Ds* transposon insertion 1022 bps or 1168 bps after the *PHE1* stop codon, respectively. The T-DNA in the insertion line (referred to as *phe1-4*) is inserted in the antisense orientation compared with the T-DNA of the *phe1-3* allele, whereas insertion of the *Ds* element (referred to as *phe1-5*) is in the same orientation as *phe1-3* (Fig. 4A). Consistent with the results obtained for the *phe1-3* allele, we observed reactivation of the maternal *PHE1* allele and a reduction of paternal *PHE1* expression (Fig. 4E and supplementary material Fig. S1C), whereas we did not observe an effect on expression of the paternal *PHE1* allele in those mutants (data not shown). Taken together, these results clearly demonstrate that the 3′ region of *PHE1* contains elements necessary for repression of the maternal *PHE1* allele.

The *PHE1* downstream region is necessary for imprinting

To obtain final proof that the identified region is necessary for *PHE1* imprinting, we designed a construct containing 3 kb of the *PHE1* promoter sequence, the *PHE1* coding region fused to a *GUS* reporter gene and a 3.5 kb sequence downstream of the *PHE1* stop codon (referred to as *PHE1*$_{3000}$*::GUS_3′*). We established transgenic plants containing this construct and tested imprinting of this

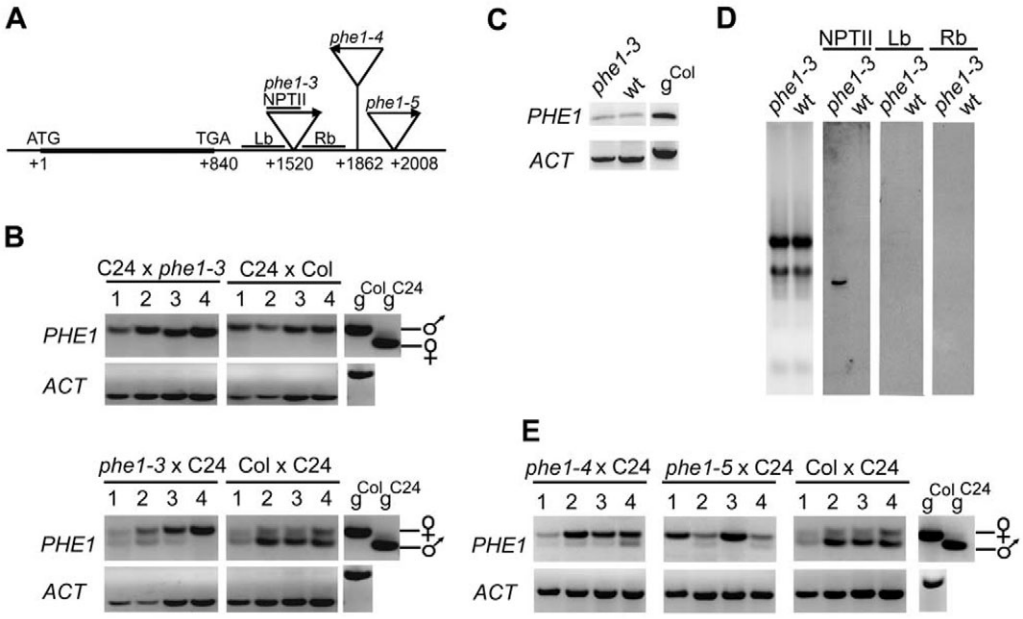

Fig. 4. Establishment of imprinting at the *PHE1* locus is compromised in mutants whose *PHE1* downstream region is disrupted. (A) Schematic overview of the location of the *phe1-3-*, *phe1-4-* and *phe1-5-* mutant alleles. Probes used for northern blot analysis are indicated. (B) Allelic expression analysis of *PHE1* after reciprocal crosses of wild-type and *phe1-3* mutant plants with the C24 accession. (C) Expression analysis of *PHE1* in leaves of wild-type and *phe1-3*-mutant plants. (D) Northern blot analysis of RNA from leaves of wild-type and *phe1-3*-mutant plants with probes indicated in panel (A). (E) Allelic expression analysis of *PHE1* in wild-type and *phe1-4-* and *phe1-4*-mutant plants after crosses of wild-type and mutant plants with the C24 accession. Lb, left border; Rb, right border; g, genomic DNA; *ACT*, *ACTIN*; wt, wild-type.

construct by monitoring allele-specific GUS expression. As imprinting of the *PHE1* transgene depends on de novo DNA methylation of the transgenic *PHE1* 3′ region, we tested whether this region indeed becomes methylated when transformed into wild-type plants. Using bisulfite sequencing we analyzed a region that allowed us to distinguish between the transgene sequence and the endogenous *PHE1* locus. As shown in Fig. 5, in this region we detected CG methylation at a level similar to that in wild type, as well as additional CNG methylation. Thus, the transgenic *PHE1* 3′ region becomes methylated de novo when transformed into wild-type plants. We tested imprinting of this transgene by performing reciprocal crosses of three independent transgenic lines with wild-type plants. Indeed, as shown in Fig. 6A, *PHE1*$_{3000}$*::GUS_3′* is exclusively expressed when inherited from the paternal parent; we did not detect expression when *PHE1*$_{3000}$*::GUS_3′* became inherited from the maternal parent. By contrast, when we tested expression of the *PHE1*$_{3000}$*::GUS* lacking the 3′ *PHE1* region, we always detected maternally derived *PHE1::GUS* expression (Köhler et al., 2005) (Fig. 6B).

Our results suggest that DNA methylation of the paternal *PHE1* allele is necessary for paternal *PHE1* expression (Fig. 2). To substantiate these findings we transformed the *PHE1*$_{3000}$*::GUS_3′* construct into a *drm1*/*drm2* mutant background. DRM1 and DRM2 are necessary for de novo cytosine methylation in all known sequence contexts and are guided to their templates by small interfering (si) RNAs (Chan et al., 2004). It has previously been demonstrated that transgene sequences when transformed into a *drm1*/*drm2* mutant background remain unmethylated owing to lack of de novo methyltransferase activity (Chan et al., 2004). Indeed, we did not detect significant levels of DNA methylation of the transgene in the *drm1*/*drm2* mutant background (Fig. 5). Using this transformation assay allowed to directly address the question whether DNA methylation of the *PHE1*$_{3000}$*::GUS_3′* transgene affects *PHE1* expression and avoids secondary effects caused by global DNA demethylation. We tested allele-specific *PHE1* expression by crossing wild-type plants with pollen from *drm1*/*drm2; PHE1*$_{3000}$*::GUS_3′* transgenic lines. In none of the three tested independent transgenic lines did we detect expression of the paternally derived *PHE1*$_{3000}$*::GUS_3′* construct (Fig. 6C). Thus, DNA methylation of the 3′ region of the paternal *PHE1* allele is necessary for *PHE1* expression. Taken together, our data clearly demonstrate that the *PHE1* downstream region contains important sequence elements necessary for imprinting of *PHE1*.

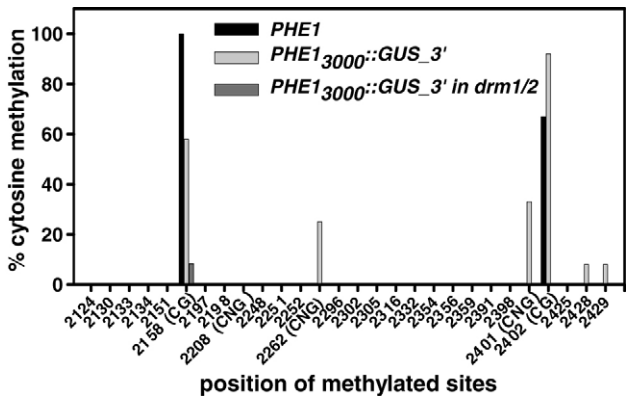

Fig. 5. Cytosine methylation profile of the *PHE1*$_{3000}$*::GUS_3′* transgene in wild-type and *drm1*/*drm2*-mutant background compared with the methylation profile of the endogenous *PHE1* locus analyzed by bisulfite sequencing. Cytosine positions relative to the translational stop codon and sequence contexts (CG and CNG, CNN not indicated) are indicated on the *x*-axis.

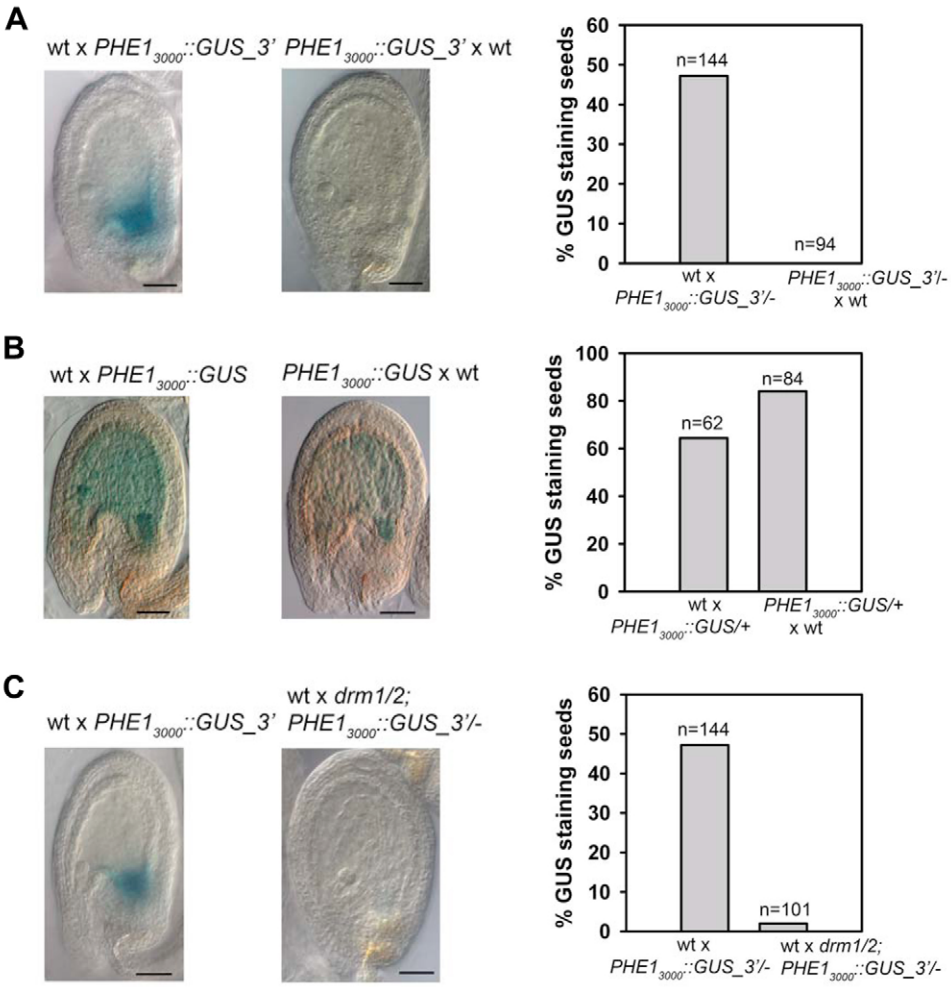

Fig. 6. The *PHE1*$_{3000}$*::GUS_3′* transgene is maternally imprinted. (A,B) Expression analysis of a maternally or paternally derived (A) *PHE1*$_{3000}$*::GUS_3′* or (B) *PHE1*$_{3000}$*::GUS* transgene in seeds at DAP3. (C) Expression analysis of a paternally derived *PHE1*$_{3000}$*::GUS_3′* transgene in a wild-type or *drm1/drm2* mutant background in seeds at DAP3. Quantification of results are shown in the respective panels on the right. Scale bars, 50 μm.

Discussion

DNA methylation is necessary for *PHE1* expression

Our data demonstrate that FIS binding to the *PHE1* promoter region and DNA demethylation of the 3′ region of *PHE1* are both necessary and sufficient for stable *PHE1* imprinting. DNA methylation in intergenic regions is often associated with transposons and repeat sequences as well as noncoding RNAs (Zhang et al., 2006). De novo methylation of repeats depends on the de novo methyltransferase DRM2 that is guided by siRNAs using the RNA-directed DNA methylation pathway (Chan et al., 2006b). Whereas DRM2-mediated de novo methylation occurs in all sequence contexts, DRM2-mediated maintenance methylation is restricted to CNG and asymmetric cytosine residues (Cao and Jacobsen, 2002). The identified direct repeats in the *PHE1* 3′ region are preferentially methylated on symmetric CG residues, indicating that methylation of *PHE1* repeats depends on MET1 maintenance methyltransferase activity. Thus, methylation of *PHE1* repeats is likely to occur through similar mechanisms as methylation of repeats in the promoter of the imprinted *FWA* gene. Methylation of endogenous *FWA* repeats depends on MET1 activity, whereas methylation of transgenic *FWA* repeats depends on DRM2 activity (Kinoshita et al., 2004; Chan et al., 2006b). However, in contrast to the role of DNA methylation for silencing of the paternal *FWA* allele, we found DNA methylation being necessary for expression of the paternal *PHE1* allele. This conclusion is supported by two findings: (1) endogenous paternal *PHE1* expression is reduced in a *met1* mutant and, (2) *PHE1*$_{3000}$*::GUS_3′* is not paternally expressed when transformed into the de novo methyltransferase mutant *drm1/drm2* that is deficient in de novo methylation of repeated transgene sequences during plant transformation (Chan et al., 2004; Chan et al., 2006b). As *PHE1*-repeat sequences are also methylated in tissues where *PHE1* is not substantially expressed, we conclude that DNA methylation is necessary but not sufficient to determine *PHE1* activity. It is possible that additional activating signals present only during seed development are necessary for *PHE1* expression.

Surprisingly, we detected expression of the maternal *PHE1* alleles in seeds inheriting a hypomethylated paternal *PHE1* allele. One possible explanation for this finding could involve recruitment of the FIS complex to the demethylated paternal *PHE1* allele. If the number of FIS complexes is limited, additional FIS target genes could cause a reactivation of silenced maternal *PHE1* alleles.

Is there a difference in DNA methylation between maternal and paternal alleles? We failed to solve this question for technical reasons, as it requires DNA isolation of either female gametophytes or pure endosperm tissues. However, given the presence of active DNA-demethylating enzymes in *Arabidopsis*, with DME having an assigned function of demethylation in the central cell, it is possible that *PHE1* becomes demethylated in the central cell of the female gametophyte. DME is necessary for *MEA* and *FIS2* expression, and lack of DME function causes seeds to abort with a fis-like phenotype. As MEA is necessary for repression of the maternal *PHE1* allele, a direct function of DME for *PHE1* regulation cannot be unequivocally addressed.

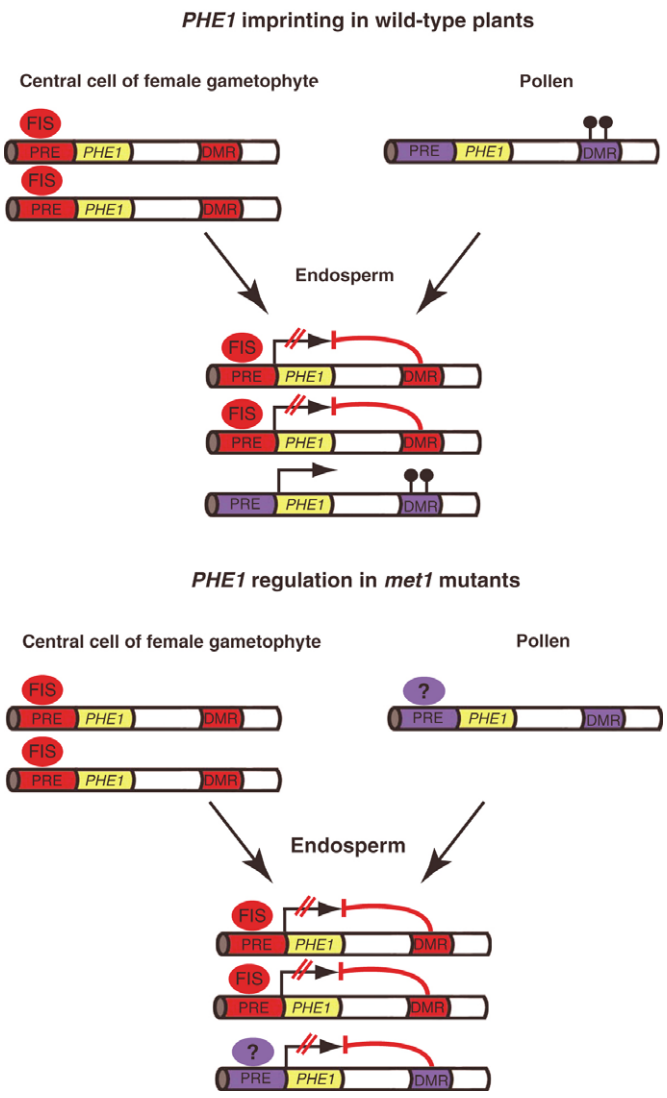

Fig. 7. Model for the establishment of *PHE1* imprinting. In the central cell of the female gametophyte the FIS-PcG complex binds to a polycomb response element (PRE) in the promoter region of *PHE1*. The differentially methylated region (DMR) is unmethylated in the central cell of the female gametophyte and blocks transcription together with the FIS complex, resulting in stable *PHE1* repression in the endosperm. The FIS complex is absent in pollen und the methylated DMR prevents silencing activity, causing the paternal allele to be active in the endosperm. In *met1*-mutant pollen, the DMR is demethylated and can block *PHE1* expression. Whether this involves PcG complexes is currently not known. Maternal alleles are shown in red, paternal alleles in blue.

Model for *PHE1* imprinting establishment

We suggest a model for *PHE1* imprinting establishment in male and female gametes (Fig. 7). We propose that the 3′ region of *PHE1* is differentially methylated, with the maternal allele being specifically demethylated in the central cell of the female gametophyte. If unmethylated, this region – together with the FIS complex – can block transcription in the endosperm either by forming a repressive chromatin loop or by facilitating the long range action of silencer regions. By contrast, in pollen, this region is methylated and either inhibits the formation of repressive chromatin loops or blocks the action of silencer elements by adopting insulator function. Both models are currently being tested. In *met1*-mutant pollen, this region is unmethylated and blocks transcription. Whether this involves the action of PcG complexes, is currently unknown. However, based on the finding that the maternal *PHE1* alleles become reactivated in seeds inheriting a hypomethylated paternal *PHE1* allele (Fig. 2B), we currently favor this hypothesis. After fertilization, the maternal *PHE1* alleles remain unmethylated in the endosperm and targeted by the FIS complex, whereas the methylated paternal *PHE1* allele is not targeted by FIS and active. This model implies that there is no reset of the *PHE1* DNA methylation imprint, because demethylation is restricted to the terminally differentiated endosperm. This suggests that maternal imprinting of *PHE1* relies on similar molecular mechanisms as paternal imprinting of *MEA*, *FWA* and *FIS2* (Xiao et al., 2003; Kinoshita et al., 2004; Jullien et al., 2006b). Our model provides an explanation for the seemingly contradictory findings that demethylation of the paternal *PHE1* allele causes paternal *PHE1* repression, whereas insertions of transgene sequences do not impair *PHE1* expression. We propose that the demethylated paternal *PHE1* allele becomes repressed, either by formation of repressive chromatin loops or the action of silencing elements. By contrast, insertions of long transgene sequences in the 3′ *PHE1* region will negatively interfere with loop formation or inhibit the long range action of silencing elements, resulting in active *PHE1* expression. We failed to detect antisense transcripts generated within the 3′ *PHE1* region in wild-type and *met1*-mutant plants (data not shown), suggesting that long antisense RNAs are not involved in *PHE1* imprinting.

Similar to the situation for *PHE1*, several imprinted genes in mammals depend on DNA demethylation for silencing, whereas DNA methylation is necessary for expression (Sleutels and Barlow, 2002). Similar to the model proposed by Sleutels and Barlow (Sleutels and Barlow, 2002), we assume that imprinting of *PHE1* evolved in two steps. First, *PHE1* expression became silenced by insertion of a repetitive sequence into a regulatory region located in the 3′ region of the gene. In a second step this repetitive sequence became methylated, thereby causing restoration of *PHE1* expression. Owing to a female-gamete-specific demethylation activity, the maternal *PHE1* allele is silenced, whereas the paternal allele is methylated and active. This model makes two predictions, (1) removal of the insertion mutation that causes the silencing effect should reactivate the silenced allele and, (2) loss of DNA methylation should result in silencing. Indeed, both predictions are supported by the results presented in this study. We could demonstrate that removal of the *PHE1* 3′ region causes de-repression of the maternal *PHE1* allele and loss of DNA methylation causes silencing of the paternal *PHE1* allele. The effect of DNA methylation to suppress the silencing effect of transposons has long been appreciated in plants (Martienssen, 1998) and was probably used as a mechanism to achieve active *PHE1* expression in male gametes.

Materials and Methods

Plant material and growth conditions

The *mea* mutant used in this study is the *mea-1* allele (L*er* accession) described by Grossniklaus et al. (Grossniklaus et al., 1998). The *met1* mutant used in this study corresponds to the *met1-3* allele (Col accession) described by Saze et al. (Saze et al., 2003). For all experiments with *met1* mutants, only first-generation homozygous plants were used. The *drm1*/*drm2* double mutant (Wassilewskija accession) was obtained from the Nottingham Arabidopsis Stock Centre (CS6366). The *cmt3-11* allele corresponds to SALK_148381 (Col accession) (Chan et al., 2006a). The mutant alleles *phe1-3*, *phe1-4* and *phe1-5* correspond to SALK_023774 (Col accession), SALK_010202 (Col accession) and GT_5_108060 (L*er* accession), respectively. The $PHE1_{3000}$*::GUS* line (L*er* accession) have been described by Köhler et al. (Köhler et al., 2003). Plants were grown in a greenhouse at 70 % humidity and daily cycles of 16 hours light at 21°C and 8 hours darkness at 18°C. Developed gynoecia were emasculated and hand pollinated one day after emasculation. For RNA expression analysis, three gynoecia or siliques were harvested at the indicated time points.

Plasmid constructs and generation of transgenic plants

To generate the $PHE1_{3000}$*::GUS_3′* construct, a 3000 bp sequence upstream of the *PHE1* translational start and the *PHE1*-coding region were amplified by PCR, introducing *Eco*RI and *Xma*I restriction sites. The fragment was ligated into pCAMBIA1381Xc, creating an in-frame fusion with the *GUS* gene. Using *Eco*RI and *Bst*EII sites, a fragment containing the *PHE1* promoter, *PHE1* coding region and the *GUS* gene was removed and introduced together with a fragment containing 3540 bps of *PHE1* 3′ region flanked by *Bst*EII and *Pst*I sites into pCAMBIA 3300. The $PHE1_{3000}$*::GUS_3′* construct was introduced into homozygous *drm1*/*drm2* plants and the corresponding Wassilewskija accession.

RNA extraction and qPCR analysis

RNA extraction and generation of cDNAs were performed as described previously (Köhler et al., 2005). Quantitative PCR was done on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems) according to the supplier's recommendations. The mean value of three replicates was normalized using *ACTIN11* as control. The primers used in this study are specified in the supplementary material (Table S1).

Allele-specific expression analysis

Allele-specific *PHE1* expression was analyzed using the assay described by (Köhler et al., 2005). The amplified products were digested with *Hph*I and analyzed on a 2.5% agarose gel. All primers are specified in the supplementary material (Table S1).

GUS expression analysis

Staining of seeds to detect GUS activity was done as described previously (Köhler et al., 2003).

Northern and Southern blot analysis

Northern and Southern blot analyses were performed as described previously (Köhler et al., 2003). Primers used to generate probes are specified in supplementary material Table S1.

Bisulfite sequencing

Bisulfite sequencing was performed following the protocol of (Jacobsen et al., 2000). Used primers are specified in supplementary material Table S1. We sequenced six clones covering the regions of 1672- 2144 bp (primers Fwd1 and Rev1) and 2099-2470 bp (primers Fwd2 and Rev2) and ten clones of the region 2425-2783 bp (primers Fwd3 and Rev3). We sequenced 12 clones covering the region 2099 bp-2470 bp (primers Fwd2 and Rev2) of transgenic lines containing the $PHE1_{3000}$*::GUS_3′* construct in wild-type and *drm1*/*drm2*-mutant background.

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