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Polycomb group proteins function in the female gametophyte to determine seed development in plants

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Polycomb group (PcG) proteins are evolutionary conserved proteins that stably maintain established transcriptional patterns over cell generations. The FERTILIZATION INDEPENDENT SEED (FIS) PcG complex from plants has a similar composition to the Polycomb repressive complex 2 from animals. Mutations in *FIS* genes cause parent-of-origin-dependent seed abortion. Every seed inheriting a mutant *fis* allele from the mother is destined to abort, regardless of the presence of a wild-type paternal allele. We tested in *Arabidopsis* whether the parent-of-origin-dependent seed abortion caused by lack of the FIS subunit MSI1 is caused by parental imprinting of the *MSI1* gene. Our data show that *MSI1* is not an imprinted gene and that early paternal *MSI1* expression is not sufficient to rescue *msi1* mutant seeds. By contrast, expression of *MSI1* in *msi1* female gametophytes is necessary to restore normal seed development, strongly arguing that the female gametophytic effect of *fis* mutants is caused by a functional requirement for an intact FIS complex in the female gametophyte. Thus, FIS-mediated expression patterns established in the female gametophyte can impact on seed development, establishing *fis* mutants as true female gametophytic maternal-effect mutants.

KEY WORDS: *Arabidopsis*, Epigenetics, *FERTILIZATION INDEPENDENT SEED* genes, Imprinting, Polycomb group proteins

INTRODUCTION

Seed development in flowering plants is initiated by a double fertilization event in which two sperm nuclei fuse with two female gametes. Male and female gametes are enclosed within multicellular gametophytes. The female gametophyte of *Arabidopsis* is embedded within the ovule and consists out of seven cells: three antipodal cells that degenerate shortly before fertilization, two synergid cells, one egg cell and one central cell. Except for the homodiploid central cell, all cells of the female gametophyte are haploid. During fertilization, the pollen tube discharges two genetically identical haploid sperm cells into the female gametophyte, where one sperm cell fuses with the egg cell, giving rise to the diploid embryo, while the other sperm cell fuses with the central cell, initiating development of the triploid endosperm (Drews and Yadegari, 2002). The embryo passes through morphologically defined stages, characterized as preglobular, globular, heart, torpedo, walking stick, early maturation and maturation (Goldberg et al., 1994; Laux and Jürgens, 1997).

Endosperm development differs dramatically from embryo development. In *Arabidopsis*, the first divisions of the primary endosperm nucleus are not followed by cytokinesis, giving rise to the formation of a syncytium. Distinct nuclear-cytoplasmic domains form: the chalazal endosperm at the posterior pole, the micropylar endosperm at the anterior pole and peripheral endosperm domains (Brown et al., 1999; Boisnard-Lorig et al., 2001). Endosperm cellularization is initiated around the globular to early heart stage of embryo development and starts in the micropylar endosperm, which surrounds the embryo, to progress through the peripheral endosperm to the chalazal region (Brown et al., 1999; Boisnard-Lorig et al., 2001). As the embryo matures, most of the endosperm is degraded and absorbed by the embryo, and only a thin aleurone layer remains.

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The endosperm is considered to support embryo growth and to regulate nutrient transfer from the mother to the developing seeds (Lopes and Larkins, 1993).

Mutations in genes of the *FERTILIZATION INDEPENDENT SEED* (*FIS*) class can form diploid endosperm in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997). Thus far, four *FIS*-class genes are known: *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), *FERTILIZATION INDEPENDENT SEED2* (*FIS2*) and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*) (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999; Ohad et al., 1999; Köhler et al., 2003a; Guitton et al., 2004). Among *fis* mutants, the *msi1* mutant has the strongest penetrance of the autonomous endosperm development phenotype (Köhler et al., 2003a; Guitton et al., 2004) and also forms parthenogenetic embryos (Guitton and Berger, 2005). The *FIS*-class genes encode proteins with homology to animal Polycomb group (PcG) proteins. Plant FIS proteins and animal PcG proteins both form multisubunit complexes with a core size of 600 kDa called Polycomb repressive complex 2 (PRC2) (Köhler et al., 2003a; Chanvivattana et al., 2004; Schwartz and Pirrotta, 2007). Animal PRC2 complexes possess histone methyltransferase activity specific for lysine 27 on histone H3 (H3K27) and possibly also H3K9 (Schwartz and Pirrotta, 2007). Similarly, plant PRC2 complexes, such as the FIS complex and the EMF2 complex, are required for H3K27 methylation and transcriptional repression of target genes (Gehring et al., 2006; Makarevich et al., 2006; Schönrock et al., 2006; Schubert et al., 2006).

Mutations in *FIS* genes cause parent-of-origin-dependent seed abortion. All seeds that inherit a mutant *fis* allele from the mother abort, regardless of the presence of a wild-type paternal allele. Development of *fis* mutant seeds is delayed and seeds abort with embryos arrested at late heart stage and displaying non-cellularized endosperm with strongly overproliferated chalazal endosperm domains (Grossniklaus et al., 1998; Kiyosue et al., 1999; Köhler et al., 2003a; Guitton et al., 2004). The maternal-effect parent-of-origin-dependent seed abortion in *mea* and *fis2* mutants can be explained by the findings that *MEA* and *FIS2* are imprinted genes, with the paternal allele of both genes being specifically silenced in

the endosperm (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Luo et al., 2000; Jullien et al., 2006a). Similarly, the paternal *FIE* allele is not expressed during early stages of seed development, providing an explanation for the maternal effect of *fie* mutants (Yadegari et al., 2000).

It is likely that MEA and FIS2 are subunits specific to the FIS complex, whereas FIE and MSI1 are part of several distinct PRC2-like complexes (Hennig et al., 2005; Schubert et al., 2005). Furthermore, MSI1 is potentially part of several different complexes, such as chromatin assembly factor CAF-1, histone deacetylases and chromatin-remodeling machines, which are likely to play a role during early embryogenesis (Hennig et al., 2005). Similar to *mea*, *fis2* and *fie* mutants, lack of MSI1 function causes parent-of-origin-dependent seed abortion. However, in addition to the gametophytic effect, it has been proposed that lack of MSI1 function also causes a sporophytic effect on seed development (Guitton et al., 2004). Thus, lack of both maternal and paternal *MSI1* alleles causes a significantly stronger defect than lack of the maternal *MSI1* allele alone. This implies that the paternal allele of *MSI1* is active, but fails to complement the maternal gametophytic *msi1* defect.

To test this idea, we investigated the temporal requirements of MSI1 during seed development. We specifically addressed the question of whether early paternal expression of *MSI1* is sufficient to rescue the maternal-effect *msi1* seed abortion phenotype. Our data clearly show that *MSI1* is not an imprinted gene and that early paternal *MSI1* expression is not sufficient to rescue *msi1* mutant seeds. By contrast, expression of *MSI1* in *msi1* female gametophytes is necessary to restore normal seed development, revealing that the female gametophytic effect of *fis* mutants is caused by a functional requirement for an intact FIS complex in the female gametophyte. Thus, FIS complex function in the female gametophyte before fertilization determines seed development after fertilization, establishing *fis* mutants as true epigenetic female gametophytic maternal-effect mutants.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of Columbia (Col) *Arabidopsis thaliana* wild-type accession were obtained from the Nottingham *Arabidopsis* Stock Centre, UK. The *msi1* allele used in this study was the *msi1-1* allele in Col described by Köhler et al. (Köhler et al., 2003a). The silent *MSI1** allele, which encodes a wild-type MSI1 protein, is a TILLING (Induced Local Lesions IN Genomes) mutant obtained from the Nottingham *Arabidopsis* Stock Centre, stock number CS92951. The Q0990, M0221 and M0223 enhancer-trap lines expressing the GFP reporter protein were generated in the laboratory of J. Haseloff (http://www.plantsci.cam.ac.uk/Haseloff) and obtained from the Nottingham *Arabidopsis* Stock Centre. The *SCR::YFP* reporter line was kindly provided by Dr B. Scheres (Utrecht University, The Netherlands). The *DR5rev::GFP* reporter line was generously given by Dr J. Friml (University of Tübingen, Germany). The *WOX8::YFP* reporter line contains 2511 bp of the *WOX8* promoter and 1775 bp of *WOX8* coding sequence fused to the *YFP* reporter and recapitulates the *WOX8* expression pattern (Haecker et al., 2004) (H.B. and T.L., unpublished). Marker lines were introduced into the *msi1* background by crossing. 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 1 day after emasculation. For RNA expression analysis, three gynoecia or siliques were harvested at the indicated time points. Dissection of seeds into embryo and endosperm plus seed coat fractions was performed under a dissection microscope. Dissected material from 100 seeds was collected in RNAlater (Ambion, Austin, USA) solution and processed as indicated below. For transmission analysis of the *msi1* mutant allele, which is tagged with a phosphinothricin resistance marker, seeds derived from reciprocal crosses were harvested 3 weeks after pollination. T1 seeds were plated on Murashige and Skoog (MS) medium containing 30 mg/l phosphinothricin, and after approximately 2 weeks the ratio of resistant to non-resistant seedlings was scored.

Plasmid constructs and generation of transgenic plants

To generate the *PHE1::MSI1* construct, the 3.0 kb of sequence upstream of the *PHE1* translational start was amplified by PCR introducing *Eco*RI and *Nco*I restriction sites. The *MSI1* cDNA was amplified by PCR introducing *Nco*I and *Bgl*II restriction sites. Both fragments were ligated into pCAMBIA 1380 using *Eco*RI and *Bgl*II restriction sites. To generate the *DD46::GUS* construct, the region 900 bp upstream of the *DD46* translational start was amplified by PCR introducing *Bam*HI and *Nco*I restriction sites and the fragment introduced into pCAMBIA 1381z using *Bam*HI and *Nco*I. To generate the *DD46::MSI1* construct, pCAMBIA 1380 was opened with *Bam*HI and *Bgl*II and the *DD46* promoter fragment flanked by *Bam*HI and *Nco*I restriction sites and the *MSI1* cDNA flanked by *Nco*I and *Bgl*II sites were introduced by ligation. All primers are listed in Table 1. Heterozygous *msi1* plants were transformed by floral dip, and transgenic plants were selected on MS medium containing 30 mg/l hygromycin. T1 plants were treated with BASTA to select for the *msi1* mutation and resistant lines were assayed for complementation of seed abortion.

RNA extraction and RT-PCR analysis

RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, USA) according to the suppliers' recommendations. For RT-PCR, total RNA was treated with 5 units of RNase-free DNase (Amersham Pharmacia Life Science, Little Chalfont, UK) for 30 minutes. Samples were extracted with phenol-chloroform and precipitated with ethanol. The RNA was reverse-transcribed using 0.5 μg of oligo dT primers (Invitrogen) in a 20 μl reaction containing 1 μl reaction buffer, 0.25 mM of each deoxynucleotide triphosphate, 5 mM dithiothreitol and 200 units of Superscript II reverse transcriptase (Invitrogen) by incubating at 42°C for 1 hour followed by heat inactivation at 72°C for 15 minutes. For RT-PCR analysis, 1/20th (by

Table 1. Primers used in this study

Amplified region	Primers (5′-3′)
ACTIN3 (*AT3G12110*)	Fwd: AACTTTCAACACTCCTGCCATG Rev: CTGCAAGGTCCAAACGCAGA
Transgene-derived *MSI1*	Fwd: GCACCGCTCTTCACACATTG Rev: TGGTCACCTGTAATTCACACG
Wild-type *MSI1*	s1: CGGTAAAGACTACTCCGTTCAGATG as1: GTAATCGAAAACATAGACCTCC
*MSI1**	s1: CGGTAAAGACTACTCCGTTCAGATA as2: GTAATCGAAAACATAGACCTCC
MSI1 cDNA	Fwd: GACCATGGGGAAAGACGAAGAGGAAATG Rev: CGAGATCTCTAAGAAGCTTTTGATGGTTC
PHERES1 promoter	Fwd: CCGAATTCGACTTTAAAATAGTAGAAAAGCTTG Rev: AATTCCATGGATCTCTTATCTTTTTCTTTTGTG
DD46 promoter	Fwd: GGATCCGGGGAAAGGAGAAAAACAAATGAGGG Rev: CCATGGTGAGCACACAAAGAGAGACGATC

volume) of the reverse-transcription samples was used to amplify cDNAs using the primers listed in Table 1. For amplification of transcripts from the *MSI1* and *MSI1** alleles from siliques, 38 PCR cycles were used; for amplification of these transcripts from dissected embryo and endosperm tissues, 40 cycles were used. For amplification of *ACTIN* transcripts, 38 cycles were always used.

Histological analysis

For detection of GUS activity, siliques were cut longitudinally, fixed for 1 hour at –20°C in 90% acetone and washed three times with 50 mM phosphate buffer (pH 7.0) before incubation at 37°C in reaction buffer (0.19 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 10 mM EDTA, 0.1% Triton X-100, 0.1 mM KFeCN, 50 mM phosphate buffer pH 7.2) for 24-72 hours. Whole seeds were observed after clearing in chloral hydrate solution (40 mM chloral hydrate, 8.3% glycerol) using a Zeiss Axioplan microscope. For GFP and YFP marker analysis, embryos from dissected seeds were mounted in deionized water. Specimens were observed under a Zeiss Axioplan microscope equipped with a GFP filter set and Nomarski optics, and images were recorded with a MagnaFire CCD camera (Optronics, Goleta, CA).

RESULTS

Loss of MSI1 causes gametophytic and sporophytic effects on seed development

Complete loss of MSI1 is lethal, and the *msi1-1* allele used in this study can only be maintained in heterozygous *msi1*/*MSI1* plants (here referred to as *msi1* plants) (Köhler et al., 2003a; Guitton et al., 2004). Self-fertilized *msi1* mutant plants form two classes of aborting seeds: an early-aborting class, which contains grossly abnormal embryos, and a late-aborting class, which contains embryos that phenotypically closely resemble *fis*-class mutant embryos (Köhler et al., 2003a; Guitton et al., 2004). It has been suggested that lack of MSI1 function has a gametophytic as well as a sporophytic zygotic effect, causing the formation of early- and late-aborting seeds, respectively (Guitton et al., 2004). This model predicts that 50% of the seeds inherit a maternal *MSI1* allele and develop normally, whereas 50% of the seeds inherit a maternal *msi1-1* allele and abort early if also inheriting a paternal *msi1-1* allele or abort late if inheriting a paternal *MSI1* allele. We observed that the *msi1-1* allele has 17% early-aborting and 33% late-aborting seeds, which deviates from the ratio of 25% early- to 25% late-aborting seeds predicted by the model [n=583, $\chi^2=29.72>\chi^2_{0.05(2)}=5.991$; Fig. 1A]. One reason for this discrepancy could be a reduced transmission of the paternal *msi1-1* allele. We tested this hypothesis by determining the transmission of the *msi1-1* allele through pollen. Indeed, we found that the transmission of the paternal *msi1-1* allele is reduced to 72% (n=500). Taking the reduced transmission of the paternal *msi1-1* allele into account, only 18% homozygous *msi1* mutant seeds can be expected. This number closely matches the observed number of 17% early-aborting seeds [50% wild type: 32% *msi1*/*MSI1*: 18% *msi1*/*msi1*; n=583, $\chi^2=0.536<\chi^2_{0.05(2)}=5.991$]. To unequivocally test the hypothesis that early-aborting seeds require a paternally inherited *msi1* allele, we pollinated heterozygous *msi1* mutant plants with wild-type pollen. In this experiment, 51% of the seeds were phenotypically wild type and 49% of the seeds were late aborting with a *fis*-like phenotype [50% wild type: 50% *msi1*/*MSI*; n=487, $\chi^2=0.166<\chi^2_{0.05(1)}=3.841$; Fig. 1B,C; Table 2]. We did not observe any early-aborting seeds, clearly proving that loss of both maternal and paternal *MSI1* alleles is the prerequisite for early seed abortion. This result suggests that the paternal *MSI1* allele is expressed and, consequently, that *MSI1* is not regulated by genomic imprinting, in contrast to the *fis*-class mutants *mea* and *fis2*.

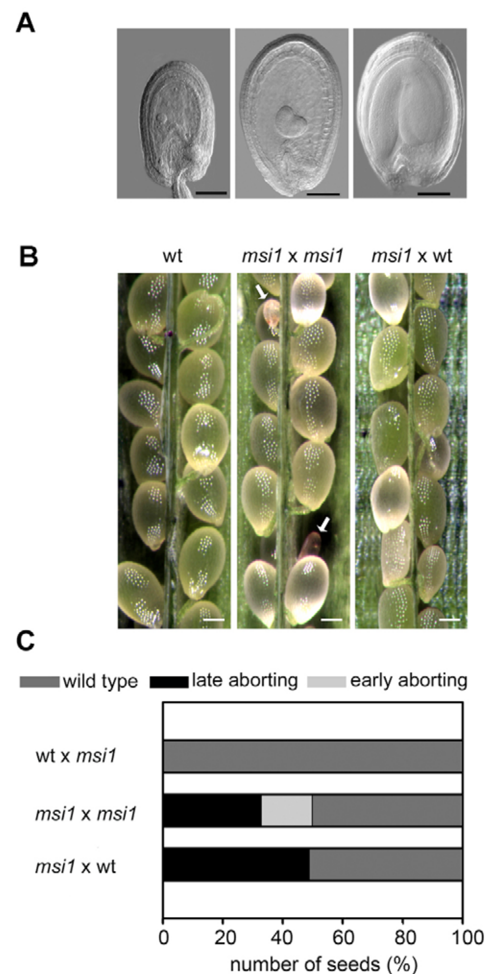

Fig. 1. Homozygous *msi1* mutant *Arabidopsis* seeds show an early developmental arrest. (**A**) Cleared seeds derived from the same silique that arrested at different developmental stages. Homozygous (left) and heterozygous (middle) *msi1* seeds and wild-type seeds (right). (**B**) Self-pollinated *msi1*/*MSI1* plants form small aborting seeds (arrows, middle). No small aborting seeds are formed after pollination of *msi1*/*MSI1* plants with wild-type pollen (right). A wild-type (wt) silique is shown as a control in the left panel. (**C**) Quantification of seed abortion observed after crosses of wt×*msi1*/*MSI1* (n=212), *msi*/*MSI1*×*msi1*/*MSI1* (n=583) and *msi1*/*MSI1*×wt (n=487). Scale bars: 50 μm in A; 200 μm in B.

MSI1 is paternally expressed in embryo and endosperm

To test the hypothesis that *MSI1* is biallelically expressed, we examined expression of maternally and paternally inherited *MSI1* alleles during early seed development. We made use of a mutant containing a silent mutation in the *MSI1* coding region (referred to as *MSI1**). We developed a PCR assay to distinguish the *MSI1** allele from the wild-type *MSI1* allele. As shown in Fig. 2A, primers s1 and as1, which were designed for the wild-type *MSI1* allele, did not amplify the *MSI1** allele. Conversely, primers s2 and as1, which were designed for the *MSI1** allele, did not amplify the *MSI1* allele. We performed reciprocal crosses between wild-type and *MSI1** plants and tested the expression of the paternally inherited *MSI1* allele. Regardless of whether wild-type plants or *MSI1** plants were used as pollen donors, we could clearly detect expression of the paternal *MSI1* allele starting 3 days after pollination (Fig. 2B). Thus,

Table 2. Analysis of seed development in self- and cross-fertilized *msi1* plants and in different transgenic backgrounds

Line	Wild type (%)	Early aborting (%)	Late aborting (%)	*n*	$\chi^2_{0.05(1)}$=3.841
wt×wt	100	0	0	269	n.a.
msi1×*msi1*	50	17	33	583	n.a.
wt×*msi1*	100	0	0	212	n.a.
msi1×wt	50	17	33	487	0.166
msi1×*msi1; PHE1::MSI1* #1	50	0	50	278	0.014
msi1×*msi1; PHE1::MSI1* #2	50	0	50	498	0.032
msi1×*msi1; PHE1::MSI1* #3	51.2	0	48.8	166	0.096
msi1; DD46::MSI1 #1×wt	100	0	0	369	n.a.
msi1; DD46::MSI1 #2×wt	100	0	0	475	n.a.

n.a., Not applicable.

the timing of paternal *MSI1* expression is comparable to that of the majority of paternal alleles (Vielle-Calzada et al., 2000). As *MSI1* is also expressed in sporophytic tissues (Hennig et al., 2003; Köhler et al., 2003a), transcripts of maternal *MSI1* or *MSI1** alleles are contributed by zygotic tissues as well as maternal sporophytic tissues. Therefore, the maternal alleles always yielded signals of higher intensity than the paternal alleles.

Imprinting of several genes has been shown to occur specifically in the endosperm, whereas the same genes are biallelically expressed in the embryo (Kinoshita et al., 1999; Kinoshita et al., 2004; Haun et al., 2007). *MSI1* is expressed in the embryo (Köhler et al., 2003a); therefore, we investigated whether expression of the paternal *MSI1* allele is confined to the embryo and is silenced in the endosperm, or whether the paternal *MSI1* allele is also expressed in the endosperm. For this purpose, we performed crosses of wild-type plants with *MSI1** plants and dissected F1 seeds at 6 days after pollination (DAP) into embryo and endosperm plus seed coat fractions. As shown in Fig. 2C, we could clearly detect expression of the paternal *MSI1** allele in the embryo as well as in the endosperm. Thus, we conclude that *MSI1* is not imprinted, but is biallelically expressed in both embryo and endosperm.

The female gametophytic defect of *msi1* mutants does not affect embryo patterning

Heterozygous *msi1* mutant seeds abort with embryos arrested at late heart stage and displaying strongly overproliferated chalazal endosperm domains (Köhler et al., 2003a; Guitton et al., 2004). However, it remains elusive why *msi1* mutant embryos arrest development and abort despite expression of the paternal *MSI1* allele. It is possible that developmental defects start to accumulate early during embryogenesis when most of the paternal genome, including *MSI1*, is still inactive and cause gross developmental abnormalities later in embryogenesis, culminating in seed abortion. Therefore, we tested whether marker genes that define major developmental steps during early embryogenesis are correctly expressed in *msi1* mutant as compared with wild-type embryos. We tested markers for auxin distribution [*DR5* (Friml et al., 2003)], the developing suspensor [*WUSCHEL-RELATED HOMEOBOX 8* (*WOX8*) (Haecker et al., 2004)], provascular tissue [enhancer-trap line Q0990 (Weijers et al., 2006)], the quiescent center [*SCARECROW* (Blilou et al., 2005)], and for cells within the region to form the shoot apical meristem [enhancer-trap lines M0221 and M0223 (Cary et al., 2002)].

The auxin-reporting *DR5::GFP* marker was confined to the root pole, cotyledon tips and provascular tissue of heart stage wild-type embryos (Fig. 3). This pattern was similar in *msi1* embryos, suggesting that auxin distribution is mostly normal in *msi1*. Expression of the *WOX8* reporter was confined to the suspensor in wild-type and *msi1* mutant embryos, indicating that the basal derivatives of the zygote forming the suspensor are correctly established. The enhancer-trap line Q0990 from the publicly available Haseloff collection (http://www.plantsci.cam.ac.uk/Haseloff/construction/catalogFrame.html) is expressed in provascular cells of the central region immediately adjacent to the hypophysis (Weijers et al., 2006). Because this expression pattern remained in *msi1*, specification of provascular cells seems to occur properly in *msi1* mutant embryos.

Establishment of root apical meristems was monitored using *SCR::YFP*, which is expressed only in the quiescent center and derivatives of the ground meristem (Wysocka-Diller et al., 2000). Expression of *SCR::YFP* in *msi1* closely resembled expression in wild type, suggesting that initiation of the root apical meristem is largely normal in *msi1*. To monitor formation of shoot apical meristems, enhancer-trap lines M0221 and M0223 were used. Both lines show GFP reporter activity in cells within the region forming the shoot apical meristem, and M0223 reflects expression of *CUP-SHAPED COTYLEDON1* [*CUC1* (Cary et al., 2002)]. As with the other markers used, activity of M0221 and M0223 was similar in wild type and *msi1*, indicating that progenitor cells for the shoot apical meristems are properly specified.

Based on these findings, we conclude that defects established in the *msi1* female gametophyte do not affect basic embryo pattern formation, and embryo arrest at late heart stage is caused by mechanisms that remain to be identified.

Paternal expression of *MSI1* immediately after fertilization cannot rescue the *msi1* female gametophytic defect

We considered two possible explanations for the female gametophytic defect of *msi1* mutants: (1) delayed expression of the paternal *MSI1* allele at only 3 DAP (Fig. 2B) is responsible for the female gametophytic defect; or (2) lack of functional MSI1 causes a defect in the female gametophyte and the consequences of this defect become obvious during later stages of seed development. We tested the first possibility by expressing paternal *MSI1* immediately after fertilization. We made use of the *PHERES1* (*PHE1*) promoter, which is one of the few promoters escaping early paternal silencing and is expressed immediately after fertilization (Köhler et al., 2005). We tested whether expression of *MSI1* under control of the *PHE1* promoter (referred to as *PHE1::MSI1*) could be detected immediately after fertilization, by crossing wild-type plants with pollen derived from *PHE1::MSI1* transgenic plants. Indeed, expression of the paternal *MSI1* allele was detected at 1 DAP (Fig. 4A). We compared the expression level of the *PHE1::MSI1* transgene with the endogenous *MSI1* allele starting at 3 DAP by semi-quantitative RT-PCR. Taking into account the different amplification efficiencies of the different primer pairs, expression of

DEVELOPMENT

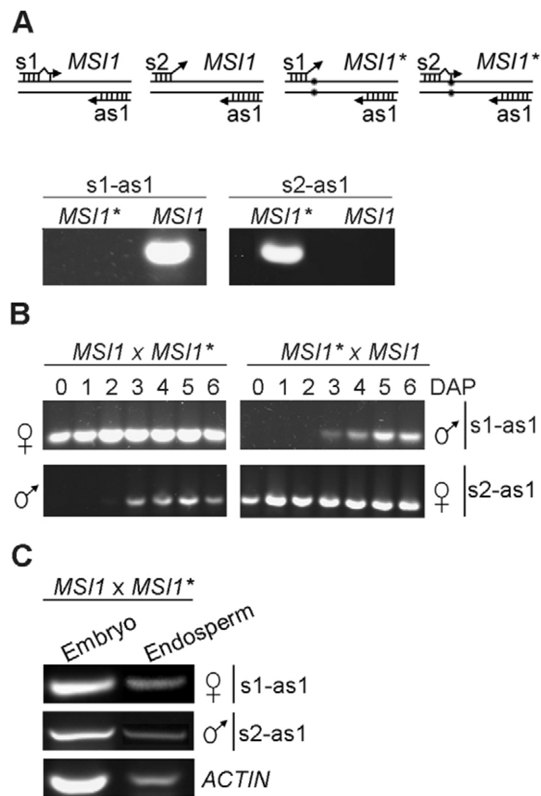

Fig. 2. The paternal *MSI1* allele is expressed in the embryo and endosperm. (**A**) Schematic of the PCR assay used to amplify specifically either the *MSI1* or *MSI1** allele. Primer combination s1-as1 amplifies only the *MSI1* allele (as shown beneath, left), whereas primer combination s2-as1 amplifies only the *MSI1** allele (beneath, right). (**B**) Time-course analysis of maternal and paternal *MSI1* expression. Reciprocal crosses of wild-type (*MSI1*) and *MSI1** *Arabidopsis* plants were performed, and expression of maternal and paternal alleles was analyzed by RT-PCR in siliques derived from these crosses. The upper panel shows results from primer combination s1-as1, the lower panel from primer combination s2-as1. (**C**) Seeds derived from a cross of *MSI* and *MSI1** plants were dissected 6 days after pollination (DAP). Embryos and endosperm plus seed coat fractions were analyzed for expression of maternal (*MSI1*) and paternal *MSI1** alleles by RT-PCR. *ACTIN* provided a positive control.

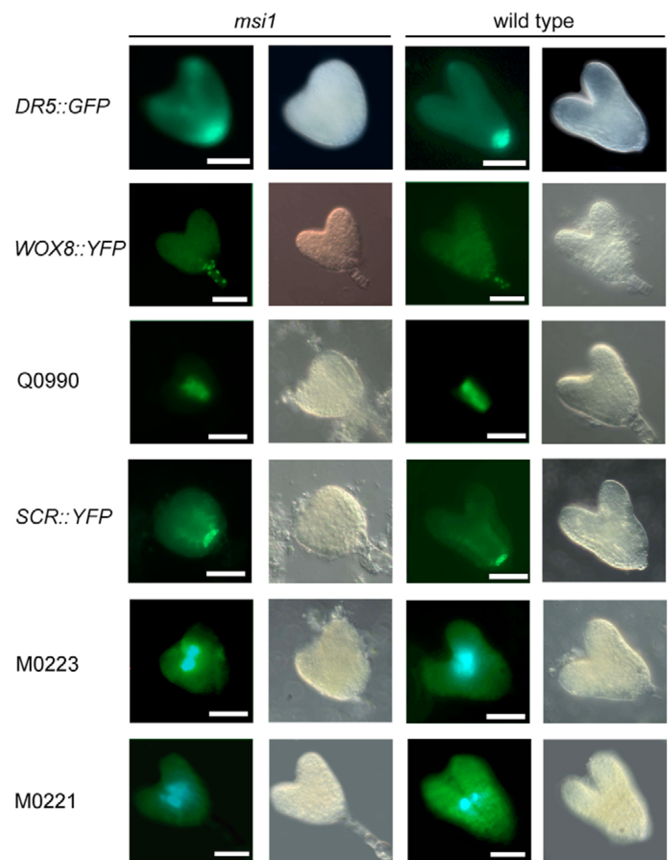

Fig. 3. Markers for embryo pattern formation are similarly expressed in wild-type and *msi1* mutant embryos. Expression of *DR5::GFP*, *WOX8::YFP*, Q0990, *SCR::YFP*, M0223 and M0221 in *msi1* mutant and wild-type *Arabidopsis* embryos. Corresponding bright-field images of embryos are shown in the right panel of each pair. Scale bars: 50 μm.

the *PHE1::MSI1* transgene was about 3.5-fold higher than that of the endogenous paternal *MSI1* allele. In heterozygous *msi1* plants, 50% of the seeds carry a maternal *msi1* allele and thus suffer from the female gametophytic defect. If early paternal expression of *MSI1* could rescue this female gametophytic *msi1* phenotype, then we would expect that a hemizygous *PHE1::MSI1* construct would rescue 25% of the seeds and lead to 75% normal seeds. However, among 11 independent transgenic *PHE1::MSI1* lines in an *msi1* mutant background, we did not identify any plant with more than 50% normal seeds, indicating that early paternal expression is not sufficient to rescue the gametophytic *msi1* mutant defect. Instead, we observed a reduction in the number of early-aborting seeds by about half, suggesting that paternally expressed *PHE1::MSI1* is sufficient to promote development of early-aborting homozygous *msi1* mutant seeds up to the stage of late-aborting heterozygous *msi1* seeds (data not shown). This hypothesis was tested by pollinating *msi1* mutant plants with pollen of three independent homozygous *PHE1::MSI1* transgenic lines in an *msi1* mutant background and scoring subsequent seed development. In contrast to pollination with pollen from heterozygous *msi1* plants, which led to 17% early-aborting seeds, after pollination with pollen from *PHE1::MSI1; msi1* transgenic lines no early-aborting seeds were observed (Fig. 4B,D; Table 2). Thus, early paternal *MSI1* expression is sufficient to establish prolonged development of homozygous *msi1* mutant seeds. We analyzed seeds of this cross by clearing and found no significant change of seed development as compared with seeds developing on *msi1* plants pollinated with wild-type pollen (Fig. 4C). To unequivocally test whether early paternal *MSI1* expression can rescue the *msi1* mutant phenotype, we tested transmission of the maternal *msi1* allele after pollination of *msi1* plants with pollen of *PHE1::MSI1* plants. We crossed *msi1* plants with pollen of three independent *PHE1::MSI* transgenic lines in a wild-type background. Using more than 100 seedlings for each line, we found no significant maternal transmission of *msi1* (Table 3). Thus, we conclude that early paternal *MSI1* expression is not sufficient to rescue the female gametophytic defect of *msi1* mutant seeds.

Expression of *MSI1* in the female gametophyte can rescue the *msi1* female gametophytic defect

Because early paternal *MSI1* expression could not rescue the female gametophytic defect of *msi1* mutants, we addressed the question of whether expression of *MSI1* in the female gametophyte could rescue

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Table 3. Transmission analysis of the *msi1* mutant allele through the female gametophyte in different transgenic backgrounds

Line	Resistant*	Sensitive	Expected (%)†
msi1×wt	0	465 (100%)	100
msi1/MSI1×*PHE1::MSI1* #1	0	138 (100%)	0
msi1/MSI1×*PHE1::MSI1* #2	1	221 (99.5%)	0
msi1/MSI1×*PHE1::MSI1* #3	1	104 (99.0%)	0
msi1/MSI1; DD46::MSI1/+ #1×wt	54	133 (71.1%)	66.7‡
msi1/MSI1; DD46::MSI1/+ #2×wt	34	123 (78.3%)	66.7‡

*The *msi1-1* allele is tagged with a phosphinothricin resistance marker and *msi1* transmission was scored by testing resistance to phosphinothricin.
†Expected percentages of sensitive seedlings.
‡In the cross *msi1/MSI1; DD46::MSI1/+* × wild type, 25% of the seeds will inherit a *msi1* mutant allele from the female, but not a *DD46::MSI1* transgene. Those seeds are expected to abort. Among the surviving seeds, 33.3% will inherit the *msi1* mutant allele together with the *DD46::MSI1* transgene and are expected to transmit the *msi1* mutation. Therefore, 66.7% of the seedlings are expected to be phosphinothricin sensitive.

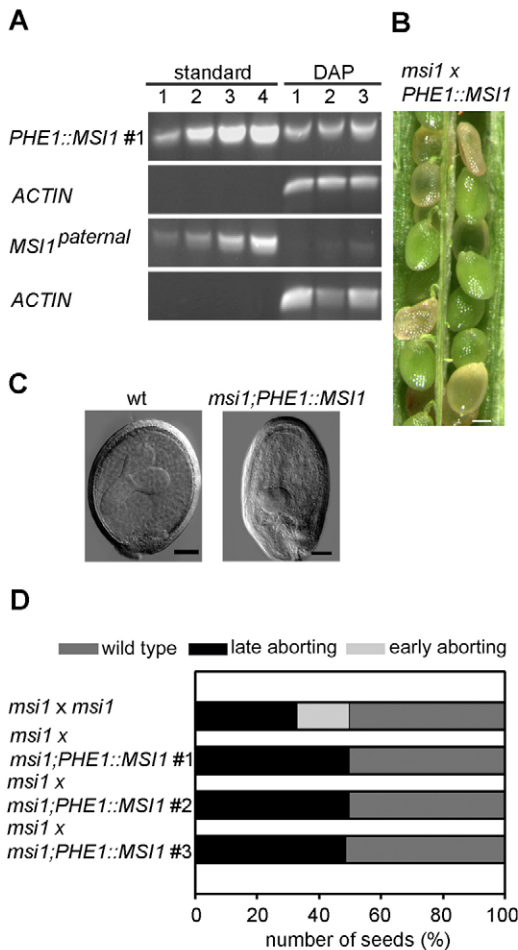

Fig. 4. Early paternal *MSI1* expression does not rescue the *msi1* mutant phenotype. (**A**) Early paternal expression of the *PHE1::MSI1* transgene was tested by RT-PCR in *Arabidopsis* seeds derived from *msi1* mutants pollinated with *PHE1::MSI1* pollen. The primers specifically detect only the transgene-derived *MSI1* transcript. Paternal *MSI1* expression in seeds derived from *MSI1** plants pollinated with wild-type pollen is shown as a control. 'Standard' refers to a dilution series of the *PHE1::MSI1* plasmid, with 1-4 containing 0.48, 2.4, 12 and 60 ng DNA, respectively. (**B**) *msi1* mutants pollinated with *PHE1::MSI1* pollen have 50% aborted seeds. (**C**) Cleared seeds derived from the same silique of an *msi1* mutant pollinated with *PHE1::MSI1* pollen. Wild-type (wt) seed (left), *msi1* mutant seed (right). (**D**) Quantification of seed abortion observed after crosses of *msi/MSI1*×*msi1/MSI1* (*n*=583) and *msi1/MSI1*×*PHE1::MSI1; msi1/MSI1* #1 (*n*=278), *msi1/MSI1*×*PHE1::MSI1; msi1/MSI1* #2 (*n*=498), *msi1/MSI1*×*PHE1::MSI1; msi1/MSI1* #3 (*n*=166). Scale bars: 200 μm in B; 50 μm in C.

the female gametophytic defect and restore wild-type seed development of *msi1*. The *DD46* promoter (At1g22015) has been shown to be active in the central cell and the synergid cells of the female gametophyte (Portereiko et al., 2006). We established transgenic plants containing the *DD46* promoter fused to the *β-GLUCURONIDASE* (*GUS*) reporter gene (referred to as *DD46::GUS*) and investigated the temporal and spatial expression pattern of this reporter construct. Before fertilization, we detected GUS activity in the central cell, in the synergids and in the egg cell. After fertilization, GUS expression ceased and was almost undetectable within the seed when the embryo had reached the globular stage, at about 2 DAP (Fig. 5A). We confirmed this expression pattern using microarray data obtained from different reproductive stages of *Arabidopsis* development (Hennig et al., 2004). Whereas *DD46* was highly expressed before fertilization, reduced transcript levels were detectable after pollination (Fig. 5B). Thus, the *DD46* promoter is specifically active in the female gametophyte and expression ceases after fertilization.

We established transgenic lines containing the *DD46* promoter fused to the *MSI1* coding sequence (referred to as *DD46::MSI1*). Using these lines, we addressed the question of whether expression of *MSI1* in female gametophytes of *msi1* mutants could rescue the seed abortion phenotype. We obtained 11 transgenic lines in an *msi1* mutant background and identified three lines with less than 50% seed abortion. Homozygous *DD46::MSI1* plants from two such transgenic lines in the heterozygous *msi1* background were pollinated with wild-type pollen and the F1 developing seeds were analyzed. We performed at least five crosses with each line, and in all instances we found a complete rescue of seed development (Fig. 5C,D; Table 2). Microscopic analysis revealed that seed development was completed without any obvious phenotypic differences to wild-type seeds (Fig. 5E). To obtain final proof that expression of *MSI1* in the female gametophyte can completely restore seed development of heterozygous *msi1* mutant seeds, we analyzed the transmission of the *msi1* mutant allele through the female gametes. We pollinated two independent transgenic lines expressing *DD46::MSI1* with wild-type pollen and tested the F1 progeny resulting from this cross for the presence of the *msi1* mutant allele. Whereas the maternal *msi1* allele was never transmitted in non-complemented mutants, most *msi1* gametophytes containing the *DD46::MSI1* construct were able to transmit the maternal *msi1* mutant allele (Table 3). Finally, we tested whether *DD46::MSI1* could suppress autonomous endosperm development in *msi1* mutants. We emasculated 13 flowers of two independent transgenic lines showing complete rescue of the *msi1* seed abortion phenotype and scored the development of the gametophytes 6 days after emasculation. Whereas the central cell of control *msi1* plants reproducibly underwent autonomous endosperm formation, all of the *msi1; DD46::MSI1* gametophytes arrested

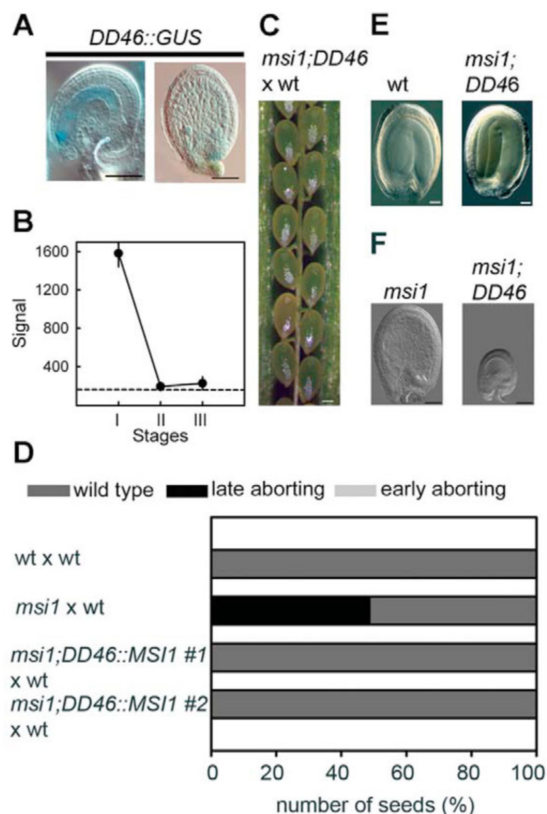

Fig. 5. Expression of *MSI1* before and shortly after fertilization can rescue the female gametophytic *msi1* mutant phenotype. (**A**) *DD46::GUS* expression can be detected in the female gametophyte (left). Residual expression of *DD46::GUS* in seeds at 2 DAP (right). (**B**) Expression of *DD46* on microarrays. *DD46* is expressed before fertilization (stage I) and is reduced to baseline (dashed line) levels after fertilization (stage II) and during seed development (stage III) [data from Hennig et al. (Hennig et al., 2005)]. (**C**) *msi1; DD46::MSI1* plants pollinated with wild-type pollen do not form aborting seeds. (**D**) Quantification of seed abortion observed after crosses of wild type (wt)×wt (*n*=269), *msi/MSI1*×wt (*n*=487), *msi1/MSI1; DD46::MSI1* #1×wt (*n*=369), *msi1/MSI1; DD46::MSI1* #2×wt (*n*=475). (**E**) Cleared seeds of *msi1/MSI1; DD46::MSI1* pollinated with wild-type pollen. Seeds of this cross (right) are indistinguishable from wild-type seeds (left). (**F**) *msi1/MSI1; DD46::MSI1* plants do not form endosperm without fertilization (right). Autonomous endosperm development in *msi1* mutants at a similar time point (left). Scale bars: 50 μm in A,E,F; 200 μm in C.

development after fusion of the polar nuclei (Fig. 5F, Table 4). Thus, we conclude that *DD46::MSI1* can completely rescue both aspects of the *msi1* mutant phenotype – seed abortion as well as autonomous endosperm development.

DISCUSSION

MSI1 has sporophytic zygotic functions

MSI1 is a subunit of the FIS PcG complex and the *msi1* mutant shares the parent-of-origin-dependent seed abortion phenotype with other mutants of the *fis* mutant class. Every seed inheriting a maternal *fis* allele aborts, regardless of the paternal contribution. However, in contrast to other *fis* mutants, *msi1* mutant seeds form two phenotypically distinguishable classes. Here, we showed that the phenotype of early seed abortion is coupled to homozygous *msi1/msi1* seeds. By contrast, seeds aborting with a *fis*-like

Table 4. Autonomous endosperm development in *msi1/MSI1; DD46::MSI1/DD46::MSI1* transgenic lines

Genotype	Seed-like (%)*	Ovules (%)	$n^{\dagger}$	Penetrance (%)‡
Wild type	0	100	324	0
msi1/MSI1	49	51	224	98
msi1/MSI1; DD46::MSI1 #1	0	100	756	0
msi1/MSI1; DD46::MSI1 #2	0	100	663	0

*Seed-like structures are defined as mainly endosperm-containing seeds developing from *msi1* mutant ovules without fertilization.
†Number of counted seed-like structures and ovules.
‡Penetrance of autonomous endosperm development phenotype.

phenotype are heterozygous *msi1/MSI1* mutant seeds derived from an *msi1* mutant female gametophyte. Besides being a member of the FIS complex and related PRC2-like complexes, MSI1 is potentially part of several other chromatin-modifying complexes (Hennig et al., 2005). A central role of MSI1 in plant development is supported by the observation that reduced MSI1 levels in *MSI1* cosuppression plants affect many aspects of sporophytic plant development (Hennig et al., 2003). Consistent with this idea is the observation that transmission of the *msi1* mutant allele through the male gametophyte is significantly reduced, suggesting that lack of MSI1 function also impairs male gametophyte development. We failed to observe a transmission defect of the *msi1* mutant allele through pollen in previous investigations (Köhler et al., 2003a). However, we noticed that transmission of the *msi1* mutant allele differs between self-pollinated and manually pollinated plants. One possible explanation for this finding is that pollen used for manual pollination is more mature than pollen of self-pollinated plants, suggesting that *msi1* mutant pollen development is delayed. Therefore, transmission analysis in this study was performed with freshly shed pollen.

In pollen of *FIE* cosuppression plants, the paternally silenced *MEA* allele becomes reactivated (Jullien et al., 2006b), suggesting that FIE is necessary for repression of *MEA* and other paternally silenced genes in pollen. It is conceivable that this repression requires a functional PRC2-like complex and that MSI1 is part of this complex. Therefore, one possible function of MSI1 during pollen development could be the repression of paternally imprinted genes like *MEA* and *FIS2*. Alternatively, MSI1 could be needed for activity of CAF-1 during pollen development. However, when testing *fas1-4* and *fas2-4* mutants (Exner et al., 2006), which lack one or other of the two CAF-1 subunits, we did not observe any transmission defect (data not shown). Future studies are needed to clarify which molecular function of MSI1 is required during pollen development. Such functions could include participation in PRC2-like complexes, in CAF-1 or in other, uncharacterized complexes.

MSI1 is biallelically expressed in the embryo and the endosperm

It has been hypothesized that the maternal effect of *fis* mutants is caused by lack of expression of paternal *FIS* alleles and, indeed, the paternal alleles of *MEA* and *FIS2* are imprinted (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Luo et al., 2000; Jullien et al., 2006a). However, our results demonstrate that this does not apply to all *fis* mutants. We show that *MSI1* is not paternally imprinted, but is clearly biallelically expressed in embryo and endosperm. The accumulation of transcripts of the paternal *MSI1* allele is delayed relative to that of the maternal *MSI1* allele. However, the timing of paternal *MSI1* expression is comparable to that of a large number of genes investigated thus far (Vielle-Calzada et al., 2000). Thus, *MSI1* is not paternally imprinted. Expression of the paternal allele of the FIS-class

gene *FIE* also occurs around 2-3 DAP, and it has been discussed that the parent-of-origin effect on seed development in *fie* and *mea* mutants is caused by different mechanisms (Yadegari et al., 2000). However, it has not been investigated whether delayed expression of the paternal *FIE* allele is responsible for the parent-of-origin effect of *fie* mutants. We tested whether delayed expression of the paternal *MSI1* allele is responsible for the *msi1* mutant phenotype by expressing *MSI1* under the control of a promoter that is paternally active immediately after fertilization. As early paternal *MSI1* expression did not rescue seed development, we conclude that MSI1 functions in the female gametophyte and establishes gene expression patterns that are required for development of the seed after fertilization. Interestingly, we also did not observe rescue of seed development when expressing the *FIS2* gene under control of the *PHE1* promoter (data not shown). In contrast to the biallelically expressed *MSI1* gene, the paternal allele of *FIS2* is not active in the endosperm; thus, *FIS2* is paternally imprinted (Luo et al., 2000; Jullien et al., 2006a). Nonetheless, early paternal expression is not sufficient to rescue the *fis2* mutant phenotype. Therefore, we conclude that paternal imprinting of *FIS* genes does not cause the parent-of-origin effect on seed development. Instead, the parent-of-origin effect of *fis* mutants is caused by lack of expression of *FIS* genes in the female gametophyte.

MSI1 activity in the female gametophyte affects seed development

PRC2-like complexes have histone methyltransferase activity, and this activity of the FIS complex appears necessary for normal seed development (Gehring et al., 2006; Makarevich et al., 2006). It is likely that genes marked by histone methylation in the female gametophyte need to be kept silent after fertilization. Indeed, the FIS target gene *PHE1* is methylated in the female gametophyte before fertilization and lack of FIS function causes strong overexpression of *PHE1* after fertilization (Köhler et al., 2003b; Makarevich et al., 2006). Thus, the FIS complex establishes epigenetic modifications on its target genes that cause stable silencing during subsequent cell divisions. This function is consistent with the proposed role of PRC2 complexes in animals to stably maintain established repressive transcriptional states (Bantignies and Cavalli, 2006). A similar function has been assigned to the PRC2-like complex containing the FIS2 homolog VERNALIZATION2 (VRN2) (Gendall et al., 2001). VRN2 is required for the vernalization-dependent stable repression of the *FLOWERING LOCUS C* (*FLC*) gene. In *vrn2* mutants, the initial repression of *FLC* after vernalization is not impaired; however, *FLC* repression is not stably maintained during subsequent periods of warm conditions (Gendall et al., 2001).

Function of the FIS complex after fertilization

All *FIS* genes are also expressed after fertilization in the endosperm (Kinoshita et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Köhler et al., 2003a), suggesting that the FIS complex has additional functions after fertilization, and it has been demonstrated that the FIS complex is necessary for suppression of the paternal *MEA* allele in the endosperm (Gehring et al., 2006; Jullien et al., 2006b). Comparing the *PHE1::MSI1* and the *DD46::MSI1* constructs, we found that expression of *MSI1* before fertilization in *msi1* mutant gametophytes is necessary to restore wild-type seed development. As *DD46* is also active after fertilization, we could not address the question of whether expression of *MSI1* in the female gametophyte is also sufficient to rescue the *msi1* maternal gametophytic defect. However, given that *FIS* genes are expressed after fertilization and that the FIS complex is functionally active, we consider this possibility as rather unlikely.

Seeds lacking a functional FIS complex have strongly overproliferated chalazal endosperm domains similar to those of seeds resulting from interploidy crosses of diploid maternal plants pollinated with pollen from tetraploid plants (Scott et al., 1998). Therefore, it has been hypothesized that the FIS complex regulates genomic imprinting and represses transcription of loci in the maternally derived genome that are normally expressed only when paternally contributed (Spielman et al., 2001). Consistent with this prediction is the expression of the FIS target gene *PHE1*, which is maternally repressed and paternally active (Köhler et al., 2005). Furthermore, pollination of *fis* mutants *mea*, *fie* and *fis2* with pollen of the *cdka;1* mutant that only forms one generative cell causes the formation of viable seeds containing a normal zygotic embryo and homodiploid endosperm. Thus, bypassing the paternal contribution can rescue *fis* mutant seeds, providing strong support for this hypothesis (Nowack et al., 2007). Therefore, it is likely that FIS complex-mediated genomic imprinting of *PHE1* and other, as yet unidentified, genes is established in the female gametophyte and is maintained by the FIS complex after fertilization.

Embryo patterning is not affected in *msi1* mutant embryos

After fertilization, the FIS complex mainly acts in the endosperm and *fis* mutants, including *msi1*, have defects in endosperm development (Grossniklaus et al., 1998; Kiyosue et al., 1999; Köhler et al., 2003a; Guitton et al., 2004). Abortion of *fis* seeds is preceded by an arrest in embryo development, and we hypothesized that defects established in *msi1* mutant gametophytes affect embryo pattern formation and cause developmental arrest of heterozygous *msi1* mutant embryos. However, all markers of early embryo development and cellular differentiation tested in this study were expressed with similar patterns in wild-type and *msi1* mutant embryos. We did not observe changes in expression of marker genes for auxin distribution, shoot and root apical meristem regions, provascular tissues and suspensor identity, indicating that there are no major defects in the establishment of the apical-basal axis or radial pattern formation. Therefore, we hypothesize that the female gametophytic defect caused by the *msi1* mutation does not directly impact on embryo pattern formation and that embryo arrest occurs by as yet undefined mechanisms. By contrast, homozygous *msi1* mutant embryos arrest development after only a few cell divisions and with severe developmental aberrations (Köhler et al., 2003a), consistent with the role of MSI1 in FIS-independent complexes.

There are two possible explanations for the developmental arrest of heterozygous *msi1* mutant embryos: (1) the arrest occurs after pattern formation by an inherent defect of the embryo; or (2) embryo arrest is caused by an external defect, i.e. in the endosperm. Several observations favor the second hypothesis. Embryo arrest of *fis* mutant seeds occurs at late heart stage. Whereas the endosperm of wild-type seeds starts to cellularize at this stage and nuclei proliferation ceases (Brown et al., 1999; Boisnard-Lorig et al., 2001), endosperm of *fis* mutants does not undergo cellularization and instead continues to divide (Kiyosue et al., 1999). The endosperm of many dicotyledonous species such as *Arabidopsis* is non-persistent and is considered as a transient medium supporting embryonic morphogenesis and early maturation by controlling the flux of nutrients delivered from the maternal plant to the developing embryo (Lopes and Larkins, 1993). The embryo is surrounded by the endosperm and both embryo and endosperm need to coordinate their development in order to produce viable seeds. Hyperproliferation of the endosperm caused by an increased paternal dosage also inhibits embryo growth, suggesting that

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increased proliferation of the endosperm is detrimental for embryo development (Scott et al., 1998). Conversely, bypassing the paternal contribution in *fis; cdka;1* double-mutant seeds restores almost wild-type-like embryo development (Nowack et al., 2007). It is conceivable that prolonged proliferation of the endosperm deprives the embryo of nutrients or, alternatively, that the endosperm does not reach the appropriate developmental stage to deliver nutrients to the developing embryo. Therefore, we suggest that lack of the FIS complex in the female gametophyte causes abnormal gene expression patterns in the central cell that persist after fertilization and produce defects in the endosperm that ultimately trigger arrest of embryo development and seed abortion.

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