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Arabidopsis MSI1 is a component of the MEA/FIE *Polycomb* group complex and required for seed development

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Seed development in angiosperms initiates after double fertilization, leading to the formation of a diploid embryo and a triploid endosperm. The active repression of precocious initiation of certain aspects of seed development in the absence of fertilization requires the *Polycomb* group proteins MEDEA (MEA), FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) and FERTILIZATION-INDEPENDENT SEED2. Here we show that the *Arabidopsis* WD-40 domain protein MSI1 is present together with MEA and FIE in a 600 kDa complex and interacts directly with FIE. Mutant plants heterozygous for *msi1* show a seed abortion ratio of 50% with seeds aborting when the mutant allele is maternally inherited, irrespective of a paternal wild-type or mutant *MSI1* allele. Furthermore, *msi1* mutant gametophytes initiate endosperm development in the absence of fertilization at a high penetrance. After pollination, only the egg cell becomes fertilized, the central cell starts dividing prior to fertilization, resulting in the formation of seeds containing embryos surrounded by diploid endosperm. Our results establish that MSI1 has an essential function in the correct initiation and progression of seed development.

Keywords: *Arabidopsis*/chromatin/MEDEA/MSI1/*Polycomb* group complex

Introduction

In contrast with animals, plant gametes do not differentiate directly after meiosis but are produced by multicellular gametophytes that develop from meiotically derived spores. In flowering plants the female gametophyte is formed through a well-defined programme of nuclear divisions, nuclear migration and cellularization that gives rise to a multicellular structure consisting of two synergids, three antipodals, a central cell and an egg cell (for review see Grossniklaus and Schneitz, 1998). Seed development is initiated by double fertilization in which two sperm cells fuse with the egg and the central cell of the female gametophyte, respectively. The fertilized egg cell develops into the embryo, while the fertilized central cell forms the nourishing endosperm. In addition, maternal tissue of the ovule, in which the female gametophyte is embedded, contributes to the developing seed by forming the seed coat (testa). Interestingly, in most angiosperms, including *Arabidopsis,* embryo and testa are diploid but the endosperm is triploid with two maternal copies and one paternal copy of the genome. Double fertilization and formation of the endosperm are key events in angiosperm evolution; however, the origin and evolutionary advantages of these two processes are not yet fully understood (for review see Chaudhury *et al*., 1998; Berger, 1999, 2003; Grossniklaus *et al*., 2001; Baroux *et al*, 2002).

After fertilization, development of testa, endosperm and embryo are highly coordinated. Genetic studies have shown that seed and fruit development are actively repressed in the absence of fertilization and that early phases of embryo and endosperm development are largely under maternal control (Vielle-Calzada *et al*., 2000; Vivian-Smith *et al*., 2001; Walbot and Evans, 2003). Several genes that encode regulators of early seed development are also required to repress fertilization-independent seed development. With reference to the Greek priestess Medea, who killed her own children in revenge for the unfaithfulness of her husband Jason, one of these genes was named *MEDEA* (*MEA*) because a mutant allele causes seed abortion only when inherited maternally (Grossniklaus *et al*., 1998). *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) and *FERTILIZATION-INDEPENDENT SEED2 (FIS2*) are two other genes that are required to repress seed development in the absence of fertilization (Luo *et al*., 1999; Ohad *et al*., 1999), a phenotype also shared by *mea* (Grossniklaus and Vielle-Calzada, 1998; Kiyosue *et al*., 1999). All three *fis*-class mutants show a gametophytic maternal effect: the mutant phenotype can only be observed when the mutation is inherited through the female gametophyte. Early seed development of *fis* mutant seeds is indistinguishable from that of wild-type seeds. However, from the globular stage onwards *fis* embryo development is delayed and eventually arrests with oversized heart-shaped embryos and an abnormally proliferated endosperm (Grossniklaus *et al*., 2001).

MEA encodes a protein similar to the *Drosophila Polycomb* group (PcG) protein Enhancer of Zeste [E(Z)] (Grossniklaus *et al*., 1998). In *Drosophila*, E(Z) interacts with ESC, a protein sharing similarity with FIE (Jones *et al*., 1998; Ohad *et al*., 1999; Ng *et al*., 2000; Tie *et al*., 2001). MEA and FIE, as well as the mammalian homologues ENX1 and EED, interact, indicating a strong evolutionary conservation of PcG proteins and their interactions (Sewalt *et al*., 1998; Luo *et al*, 2000; Spillane *et al*., 2000; Yadegari *et al*., 2000). The E(Z)–ESC complex has a molecular mass of about 600 kDa and

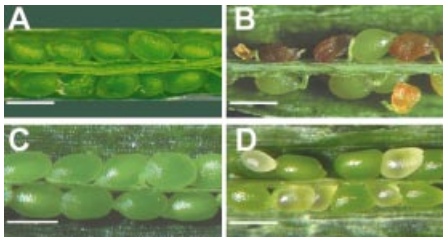


Fig. 1. Siliques of *MSI1/msi1* plants contain 50% normal and 50% aborted seeds. Opened siliques of (**A**) wild-type (WT), selfed, (**B**) *MSI1/msi1*, selfed, (**C**) WT × *MSI1/msi1* and (**D**) *MSI1/msi1* × WT. Scale bars, 500 μm.

contains additional subunits, including p55 and the FIS2 homologue Su(Z)12 (Luo *et al*., 1999; Tie *et al*., 2001; Müller *et al*., 2002). Because many SET domain proteins like E(Z) have histone methyltransferase activity, it has been proposed that PcG complexes establish repressive chromatin environments at certain target loci through histone deacetylation and histone methylation (for review see Francis and Kingston, 2001). It is now increasingly appreciated that maintenance of intact chromatin states is critical for normal development and the maintenance of developmental decisions (for review see Muller and Leutz, 2001; Köhler and Grossniklaus, 2002; Reyes *et al*., 2002; Reyes and Grossniklaus, 2003).

WD-40 proteins similar to yeast MSI1 have key functions in the maintenance and modulation of chromatin. These proteins exist in all eukaryotes and participate in various complexes involved in chromatin dynamics, including the above mentioned PcG complexes, as well as chromatin assembly factor CAF-1, nucleosome remodelling factor NURF, histone acetyl transferase and histone deacetylase complexes, and they interact with the retinoblastoma tumor suppressor protein (Qian *et al*., 1993; Parthun *et al*., 1996; Taunton *et al*., 1996; Verreault *et al*., 1996). Members of this family include p55 in *Drosophila*, the retinoblastoma-associated proteins RbAp46 and RbAp48 in vertebrates and CAC3p in *Saccharomyces cerevisiae*. Despite their ubiquitous presence, very little is known about the *in vivo* function of MSI1-like proteins. In yeast, CAC3p is required for efficient gene silencing at telomeres and the mating type loci, and *cac3* mutants show enhanced sensitivity to ultraviolet radiation (Kaufman *et al*., 1997). In *Caenorhabditis elegans*, dominant negative alleles of the MSI1-like *LIN-53* gene cause defects in vulva differentiation (Lu and Horvitz, 1998), but no other functions of MSI1-like proteins in multicellular eukaryotes have been reported. *Arabidopsis* has five *MSI1*-like genes (*MSI1–5*) (Ach *et al*., 1997; Kenzior and Folk, 1998; Hennig *et al*, 2003), and reducing *Arabidopsis* MSI1 levels by co-suppression causes ectopic expression of floral homeotic genes and strongly interferes with cellular differentiation (Hennig *et al*., 2003). Because the severity of phenotypic alterations increased during development, we suggested that MSI1 is required for the inheritance of epigenetic states during mitosis.

Here we describe an *Arabidopsis MSI1* insertion allele that causes seed abortion early in development when maternally inherited. Heterozygous *msi1* mutants also show a high penetrance of fertilization-independent seed development. We demonstrate that *Arabidopsis* MSI1 physically interacts with FIE and that MSI1, FIE and MEA are part of a 600 kDa protein complex *in vivo* that is required for seed development.

Table I. Seed abortion in *msi1* and *fasciata* mutants

	Normal	Aborted	Expected	*p* value
WT	549	1 (0.2%)	NA	NA
MSI1/msi1 × *MSI1/msi1*	268	261 (49.3%)	50	0.76
fas1	162	2 (1.2%)	NA	NA
fas2	176	4 (2.2%)	NA	NA
msi1 compl-1	184	66 (26.4%)	25	0.61
MSI1/msi1 × *MSI1/MSI1*	93	99 (48.4%)	50	0.66
MSI1/MSI1 × *MSI1/msi1*	262	0 (0.0%)	0	NA

NA, not applicable.

Results

Identification of an msi1 T-DNA insertion mutant

To analyse the function of *Arabidopsis MSI1* we searched several collections of T-DNA insertion mutants for a disruption of the *MSI1* gene and identified one candidate in the SAIL collection (Sessions *et al*., 2002). Genomic DNA blots revealed a complex insertion of two T-DNAs at the same locus. PCR analysis and sequencing confirmed the insertion into the second exon of the *MSI1* gene (data not shown). Heterozygous plants developed normally from the seedling stage to maturity. The T-DNA insertion allele in *Arabidopsis MSI1* was termed *msi1* and was used in all subsequent experiments.

Loss of MSI1 causes a maternal effect seed abortion phenotype

We were unable to obtain homozygous *msi1* plants, suggesting that seed development is defective in *MSI1/msi1* mutants. In contrast with wild-type plants that contained only normally developing seeds, *MSI1/msi1* siliques had aborted seeds that appeared brown and shrunken (Figure 1). Quantification of seed abortion revealed that 50% of the seeds from heterozygous *msi1* mutants aborted, in contrast with less than 1% abortion observed in the wild type (Table I). *Arabidopsis* MSI1 was previously reported to participate in CAF-1 (Kaya *et al*., 2001), but we found no striking overlapping phenotypes between the *Arabidopsis* CAF-1 mutants *fasciata1* (*fas1*) and *fasciata2* (*fas2*) and *MSI1* co-suppression plants (Hennig *et al*., 2003). Therefore we assayed seed abortion in *fas1* and *fas2*, but, in contrast with *MSI1/msi1* plants, seed development was not impaired in the CAF-1 mutants (Table I).

A single-locus recessive embryo-lethal mutant is expected to give rise to 25% aborted seeds. However, seed abortion in *MSI1/msi1* occurred at a ratio of 50%. Because 50% seed abortion is a strong indication for a defect that is under female gametophytic control, we performed reciprocal crosses between *MSI1/msi1* and wild-type plants. A paternally derived mutant allele (*msi1* pollen) did not impair seed development, while the *msi1*

Table II. Transmission of the *msi1* allele

	Phosphinotrocin sensitive	Phosphinotrocin resistant	Expected	*p* value
MSI1/msi1 × *MSI1/msi1*	113	125 (52.5%)	50	0.44
			75	< 0.01
MSI1/MSI1 × *MSI1/msi1*	42	44 (51.1%)	50	> 0.05
MSI1/msi1 × *MSI1/MSI1*	51	0 (0.0%)	0	NA
msi1 compl-1 × *MSI1/MSI1*	48	26 (36.8%)	33	0.696

NA, not applicable.

phenotype could not be complemented by fertilization of *msi1* mutant gametophytes with wild-type pollen (Figure 1 and Table I). These observations suggest that the fate of seeds is determined only by the maternally derived *MSI1* allele. Seeds derived from ovules with an *msi1* gametophyte (hereafter referred to as *msi1* seeds) abort regardless of the paternal genotype. This hypothesis predicts that the *msi1* mutant allele can be transmitted only paternally. We tested for the presence of the *msi1* allele in the progeny of selfed *msi1* heterozygous plants and of reciprocal crosses between *MSI1/msi1* and wild-type plants. The results shown in Table II confirm this hypothesis.

Genetic complementation of msi1

T-DNA insertion mutagenesis can also result in mutations that are not tagged by the inserted T-DNA (Azpiroz-Leehan and Feldman, 1997). Therefore we tested whether the observed *msi1* phenotype is indeed caused by the insertion into the *MSI1* gene. Plants heterozygous for *msi1* were transformed with the *MSI1* cDNA under control of the endogenous *MSI1* promoter consisting of a 2 kbp DNA fragment upstream of the translation start codon. Two randomly chosen transgenic lines showed less seed abortion than the parental *MSI1/msi1* mutant. We selected a transgenic line with a single insert of the transgene (*msi1 compl-1*, data not shown) for further analysis. As *msi1 is* a gametophytic maternal effect mutant, the expected ratio of aborted seeds in an *MSI1/msi1* plant is 50%. An unlinked single copy *MSI1* transgene will segregate randomly in the gametes such that half of the *msi1* seeds are expected to contain the transgene rescuing the *msi1* phenotype. This will reduce the ratio of aborted seeds to 25% (see below). As expected, the fraction of aborting seeds dropped from 49.3% in *MSI1/msi1* to 26.4% in *MSI1/msi1 compl-1* (Table I). Because seed abortion can be complemented by an intact *MSI1* cDNA, we conclude that this phenotype of *MSI1/msi1* plants is indeed caused by loss of *MSI1* function.

Aborting seeds in msi1 arrest early during embryo development

We analyzed the morphology of mutant and wild-type seeds in cleared whole-mount and semi-thin sectioned specimens to characterize the defects of *msi1* mutant seeds in more detail (Figure 2). Mutant *msi1* seeds were developmentally delayed compared with their wild-type siblings. The developmental arrest occurred at different developmental stages and was highly variable among different siliques. Thus, when about half of the seeds of *MSI1/msi1* plants contained transition or heart-stage embryos (Figure 2A and D), about 36% (n = 361) of the seeds contained preglobular and globular stage embryos (Figures 2B and E). The remaining seeds aborted shortly after fertilization without the formation of any embryo. In contrast with the well-organized structure of preglobular embryos in wild-type seeds (Figure 2C and F), delayed preglobular seeds in *MSI1/msi1* siliques often contained abnormal embryos with irregular orientation of cell division planes. In fact, there was no recognizable separation between the embryo proper and the suspensor (Figure 2B and E). Very few *msi1* embryos continued development until the heart stage. However, *msi1* heart-stage embryos showed overproliferation and the seeds contained an enlarged chalazal endosperm (Figure 2H and L). When developing wild-type seeds had reached the late torpedo stage (Figure 2G and K), the majority of the mutant siblings were already aborted and only very few contained overproliferated heart-stage embryos. Consequently, seed development is initiated in *msi1* female gametophytes but suffers from early defects in embryo and endosperm development, eventually leading to seed abortion.

MSI1 is strongly expressed in the female gametophyte and the embryo

MSI1 is strongly expressed in floral buds and flowers (Hennig *et al*., 2003), but seed abortion in the absence of an intact maternal *MSI1* allele suggested expression of *MSI1* in fruits also. RT-PCR analysis demonstrated that *MSI1* is strongly expressed in siliques during wild-type seed development from 0 to 4 DAP (Figure 3A). In order to characterize the tissue-specific *MSI1* expression, we performed *in situ* hybridization experiments (Figure 3B). We obtained a strong signal with the antisense RNA probe in both the female gametophyte and the sporophytic tissue of the ovules (e.g. in integuments). After fertilization, the strongest expression was detected in developing embryos. We also observed specific hybridization signals in pollen sacs and pollen (data not shown). Together, these results support the view that *MSI1* has a pivotal role in gametophyte and embryo development.

MSI1, MEA and FIE participate in a high molecular weight complex in vivo

Similar to *msi1*, the maternal effect mutants *mea*, *fie* and *fis2* also cause 50% seed abortion. Since MEA and FIE interact *in vitro* and *in vivo* (Luo *et al*., 2000; Spillane *et al*., 2000; Yadegari *et al*., 2000), we tested whether MSI1 is also a subunit of the MEA–FIE complex. In *Drosophila*,

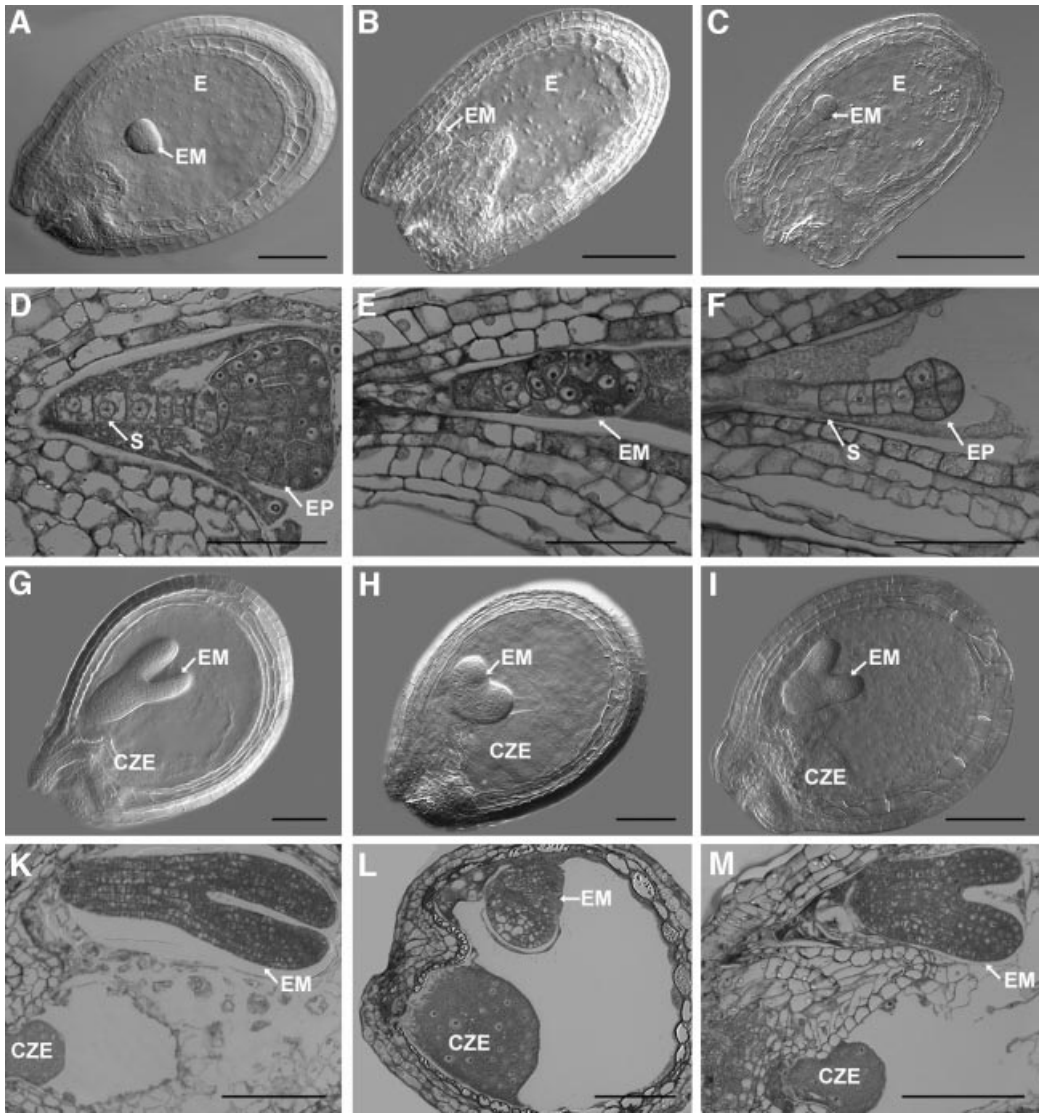


Fig. 2. Mutant *msi1* seeds contain abnormal embryos arrested at different developmental stages: (**A**), (**D**) Wild-type seeds with transition stage embryos; (**B**), (**E**) mutant seeds from the same silique as in (A) and (D); (**C**), (**F**) preglobular wild-type embryos for comparison; (**G**), (**K**) wild-type seeds with torpedo stage embryos; (**H**), (**L**) mutant seeds from same silique as (G) and (K) containing enlarged mutant embryo at the heart stage and chalazal endosperm that is overproliferated; (**I**), (**M**) wild-type seeds with late heart-stage embryos for comparison. Abbreviations: CZE, chalazal endosperm; E, endosperm; EM, embryo; EP, embryo proper; S, suspensor. Scale bars, 100 μm in (A), (B), (C), (G), (H), (K), (I) and (M); 50 μm in (D), (E), (F) and (L).

E(Z) and ESC are subunits of a large complex with a molecular mass of about 500–600 kDa (Ng *et al*., 2000; Tie *et al*., 2001). In *C.elegans*, however, homologues of E(Z) and ESC are found in a complex with a molecular weight of only 255 kDa (Xu *et al*., 2001). The molecular weight and subunit composition of the *Arabidopsis* MEA–FIE complex is currently unknown. Therefore we investigated the MEA–FIE complex using size exclusion chromatography (SEC) of protein extracts from *Arabidopsis* flowers and young siliques and tested the fractions on protein blots. The antisera used in these experiments had previously been shown to recognize the corresponding antigen and do not cross-react with other proteins of the same size (Hennig *et al*., 2003; Köhler *et al.*, 2003). Figure 4A shows that MSI1, MEA and FIE co-elute at a molecular weight of about 600 kDa, suggesting that they are part of a complex similar to the PcG complex in *Drosophila*. Interestingly, a large amount of FIE, but not MEA and MSI1, could also be detected in a monomeric form. To confirm that MSI1 is also a subunit of the MEA–FIE complex, we performed immunoprecipitation experiments with *Arabidopsis* nuclear extracts. Both anti-MEA and anti-FIE, but not the preimmune sera, coprecipitated MSI1 (Figure 4B).

To characterize the protein–protein interactions between MSI1, MEA and FIE in more detail, an MSI1–GST fusion protein was expressed in *Escherichia coli* and incubated with MEA or FIE proteins before binding to gluthathione beads (Figure 4C). The binding assay revealed that MSI1 can efficiently bind to FIE, but not to MEA, when it is presented as a single binding partner. These results suggest that MSI1 and FIE interact directly *in vivo*, but that the interaction of MEA and MSI1 requires either post-translational modifications of at least one partner or additional proteins like FIE to mediate the interaction.

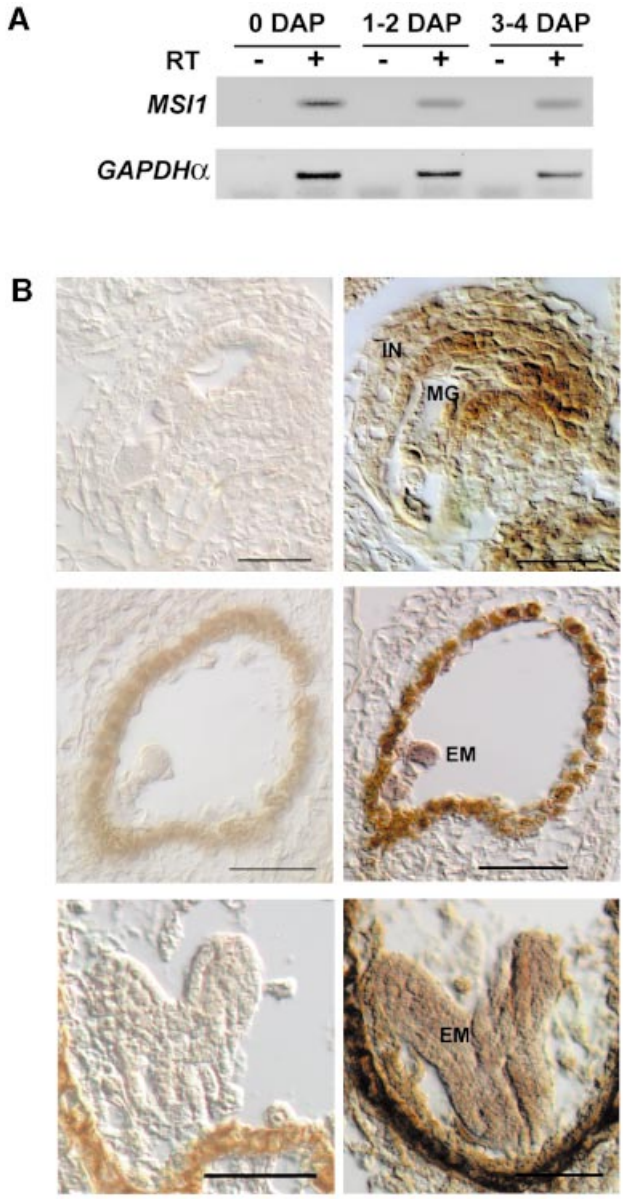


Fig. 3. *MSI1 is* expressed in the female gametophyte and during different stages of seed development. (**A**) RNA was isolated from wild-type flowers before fertilization (0 DAP), siliques containing embryos at preglobular stage (1–2 DAP) and siliques containing embryos at late globular stage (3–4 DAP). After treatment with DNase I, RNA was subjected to reverse transcription in the presence (+) or absence (–) of reverse transcriptase using oligodT primers. PCR with cDNA-specific primers was performed on aliquots of the produced cDNA, which equalled 50 ng total RNA. (**B**) Localization of *MSI1* expression in gametophytes and developing seeds of wild-type *Arabidopsis* plants. Sections were hybridized with a sense (left) or antisense (right) *MSI1* probe. Top, expression in female gametophytes; middle, expression in globular embryos; bottom, expression in heart-stage embryos. Abbreviations: EM, embryo; MG, megagametophyte; IN, integuments. Scale bars: top, 30 μm; middle and bottom, 50 μm.

Loss of MSI1 leads to fertilization-independent seed development

Both *mea* and *fie* belong to the *fis* class of mutants, and the current model suggests that the MEA–FIE complex prevents seed development in the absence of a fertilization signal (for review see Grossniklaus *et al*., 2001). If MSI1 is an integral part of the MEA–FIE complex, we would also expect autonomous endosperm or seed development in *MSI1/msi1* mutants. Floral buds of wild-type and *MSI1/msi1* mutant plants were emasculated and compared with manually pollinated gynoecia. Pollinated gynoecia of wild-type and *MSI1/msi1* mutant plants were indistinguishable after 6 days and reached a length of about 13 mm. In contrast, after 6 days without pollination, gynoecia of wild-type plants were only 2–3 mm long, whereas in *MSI1/msi1* mutant plants unpollinated gynoecia formed siliques of 10 mm (Figure 5).

Since silique elongation in the absence of fertilization suggests autonomous endosperm and seed development we investigated the developing seeds in *MSI1/msi1* plants in more detail. Figure 6 shows that unfertilized wild-type ovules did not develop (Figure 6B), whereas many unfertilized *msi1* ovules initiated seed development (Figure 6C and D). Optical and semi-thin sections through such fertilization-independent seeds revealed the presence of a multinuclear endosperm but no embryo. Sometimes embryo-like structures could be observed at the micropylar end of the embryo sac (Figure 6D). However, in contrast with embryonic cells that contain small vacuoles, these cells were highly vacuolated, suggesting that they originated from the endosperm. Only 50% of the ovules carry a mutant *msi1* allele and thus can be expected to initiate fertilization-independent seed development. Quantification revealed that 41.2% of the ovules displayed the FIS phenotype, demonstrating a penetrance of more than 80%. This is significantly higher than the reported penetrance of 15–20% in the *mea* mutants (Grossniklaus and Vielle-Calzada, 1998; Kiyosue *et al*., 1999).

Double fertilization is impaired in msi1

The high penetrance of the FIS phenotype in *MSI1/msi1* plants suggests that endosperm development fails to arrest and starts without fertilization even after pollination. We tested this hypothesis by performing ploidy analysis using flow cytometry of nuclei from developing seeds (Figure 7). This assay was previously used to demonstrate that endoreduplication produces 6C and 12C nuclei in the endosperm and 4C, 8C and 16C nuclei in the embryos of several species (Matzk *et al*., 2000). We observed up to 40% 3C or 6C nuclei derived from the endosperm in wild-type seeds at 6 DAP. As expected, seeds developing in unpollinated *MSI1/msi1* siliques did not yield any 3C or 6C signal, demonstrating that these seeds contain only nuclei derived from the diploid central cell. Surprisingly, 3C or 6C signals were also largely absent in *msi1* mutant seeds derived from pollinated gynoecia. The small percentage of 3C and 6C nuclei detected in some preparations (compare Figure 7A, bottom, and B) suggests that double fertilization can occur in a small fraction of *msi1* seeds. Because we observed embryo formation in *msi1* mutant seeds only after pollination, fertilization of the egg cell appears to be required for development of the embryo but not the endosperm.

Discussion

MSI1-like proteins are components of various chromatin-modifying protein complexes, but their biological function is not well understood at present. In yeast, MSI1 is required for gene silencing at telomeres and mating type

loci (Kaufman *et al*., 1997; Enomoto and Berman, 1998). In *C.elegans*, LIN53 is repressing genes required for the development of vulval cell fates (Lu and Horvitz, 1998). Recently, we have shown that *Arabidopsis* MSI1 is required for the maintenance of differentiation processes in vegetative and reproductive development (Hennig *et al*., 2003). In many organisms, including yeast, vertebrates and many plants, several MSI1-like proteins exist that are functionally distinct (Verreault *et al*., 1996; Ach *et al*., 1997; Kaufman *et al*., 1997; Hennig *et al*., 2003). However, insects like *Drosophila* and *Anopheles* contain only a single *MSI1*-like gene. Sequence similarities suggest that *MSI1*-like genes in different clades diverged independently during evolution. However, the advantages or costs of MSI1 diversification are not well understood. The *Arabidopsis* genome encodes five MSI1-like proteins (MSI1–5). *MSI2* and *MSI3*, as well as *MSI4* and *MSI5*, are pairs of closely related genes, while *MSI1* is more distantly related and has no close homologue (Hennig *et al*., 2003). We isolated an insertion mutant in the *MSI1* gene that is most likely a complete knockout of MSI1 function. Heterozygous mutant plants developed normally until the seed set. This observation is consistent with the fate of *msi1* antisense plants, which contain about 30% of wild-type MSI1 protein levels but have a normal morphology (unpublished results). Only the reduction of MSI1 protein content to about 5% of wild-type levels in *msi1* co-suppression plants caused severe defects during vegetative and reproductive development (Hennig *et al*., 2003). Seed abortion in heterozygous *msi1* insertion mutants demonstrated a central function of *MSI1* in seed development that was not uncovered in the co-suppression plants.

The maternal MSI1 allele is required for seed development

Similar to the loss-of-function alleles of *MEA*, *FIE* and *FIS2*, loss of MSI1 function also has a gametophytic maternal effect. Reciprocal crosses demonstrated that wild-type seed development occurs in the presence of a maternally inherited *MSI1* allele, but seeds that contain a maternally derived *msi1* allele abort their development regardless of the paternal contribution. Maternal effects can occur for a variety of reasons (Grossniklaus and Schneitz, 1998; Grossniklaus *et al*., 1998), including

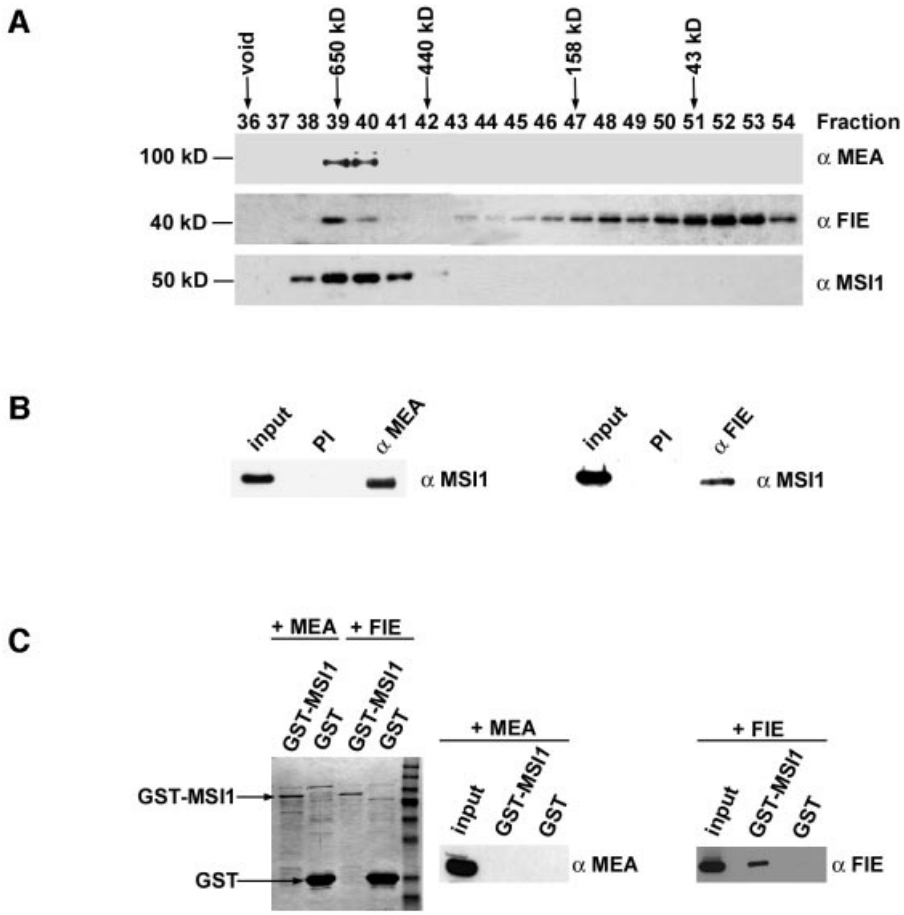
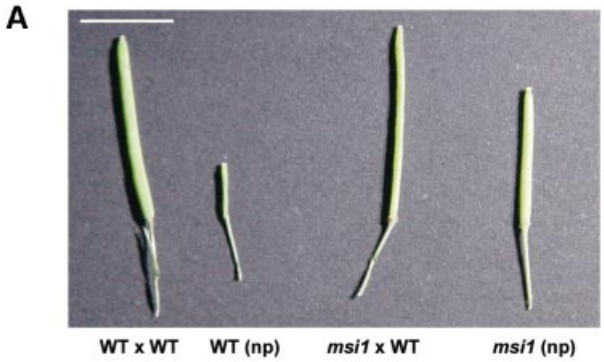


Fig. 4. MEA, FIE and MSI1 are part of a large protein complex. (**A**) Gel filtration analysis of nuclear extracts of plant inflorescences. Nuclear extracts were loaded onto a 14 ml Bio-SEC-250 column. Fractions were separated by SDS–PAGE and tested on protein blots. Fraction numbers are indicated at the top and arrows indicate elution positions of molecular mass standards. (**B**) MSI1, MEA and FIE reside in one protein complex *in vivo*. Co-immunoprecipitations were performed on nuclear extracts prepared from plant inflorescences. Immunoprecipitated proteins were analyzed on protein blots with the antibodies indicated at top. Input contains 3% of the protein used for the co-immunoprecipitation assay. (**C**) MSI1 and FIE interact physically *in vitro*. Bacterial extracts containing GST-MSI, MEA or FIE were mixed, incubated together and bound to glutathione beads. GST alone was used as a negative control. After extensive washing, proteins were analyzed by SDS–PAGE and Coomassie staining (left panel) or protein blotting (right panel). Input contains 4% of the protein used for the pulldown assay.

dosage effects in the endosperm, monoallelic expression after fertilization (genomic imprinting) or by affecting a stored gene product that is produced prior to fertilization but is required during seed development. In the triploid endosperm a single wild-type allele delivered by the pollen might not suffice to compensate two maternally derived mutant alleles. However, pollination of *MSI1/msi1* plants with pollen of a tetraploid wild-type plant did not change the fraction of aborting seeds despite the presence of two wild-type *MSI1* alleles (data not shown). Similarly, plants that were homozygous for the endogenous *MSI1* wild-type allele and for a complementing *MSI1* transgene carry two *MSI1* copies in their gametes, but pollination with pollen from these plants did not affect seed abortion ratios in *MSI1/msi1* plants (data not shown). Parent-of-origin effects can also be due to maternal or paternal allele-specific differential expression (genomic imprinting) (Grossniklaus *et al*., 2001). It was shown that the paternal alleles of the *FIS* genes are not expressed after fertilization, indicating that they are regulated by genomic imprinting (Vielle-Calzada *et al*., 1999; Luo *et al*., 2000). Further studies will be necessary to test whether the parent-of-origin effect of *msi1 is* due to a paternally silenced *MSI1* allele or the deficiency in the cytoplasmic store of the MSI1 protein. The seed arrest phenotype of *mea* and *fis2*, but not *fie*, is alleviated by pollination with a low-methylation parent or the introduction of the *ddm1* mutation, which is deficient in a putative chromatin remodelling factor and reduces DNA methylation (Jeddeloh *et al*., 1999; Vielle-Calzada *et al*., 1999; Luo *et al*., 2000; Yadegari *et al*., 2000). In contrast, we did not observe higher fractions of developing seeds in selfed *MSI1/msi1*; *DDM1/ddm1* plants (data not shown), indicating that defects caused by the loss of *MSI1* activity cannot be rescued by activating silenced genes. Together, our data show that a maternal copy of *MSI1 is* essential for successful seed development.



B

	Silique length
MSI1 MSI1 x *MSI1 MSI1*	12.8 ± 0.4 mm
msi1 MSI1 x *MSI1 MSI1*	14.1 ± 0.8 mm
MSI1 MSI1	2.5 ± 0.1 mm
msi1 MSI1	9.9 ± 0.5 mm
msi1 MSI1 compl.-1	4.7 ± 0.4 mm
fas1	2.7 ± 0.3 mm
fas2	1.7 ± 0.3 mm

Fig. 5. *MSI1/msi1* plants undergo fertilization-independent silique elongation. (**A**) Representative siliques of wild-type and *MSI1/msi1* plants at 6 DAP or non-pollinated (np). Scale bar, 5 mm. (**B**) Average silique length of wild-type or *MSI1/msi1* plants (mean ± SE, $n = 10$) at 6 DAP or 6 days after emasculation (*MSI1/msi1*).

Loss of MSI1 function relaxes endosperm development in the absence or presence of fertilization

In most plants, seed and fruit development are actively repressed and usually initiate only after fertilization.

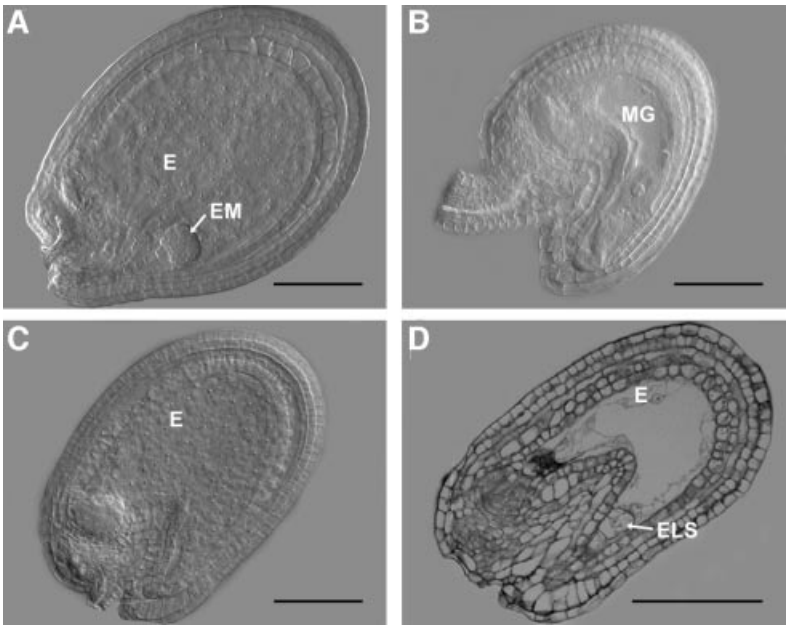


Fig. 6. Fertilization-independent seed development in *MSI1/msi1* plants. (**A**) Wild-type seed at 6 DAP containing a dermatogen-stage embryo. (**B**) Unfertilized wild-type ovule at 6 DAP. (**C**), (**D**) Unfertilized *msi1* ovules that started seed development and contain multinuclear endosperm. Abbreviations: E, endosperm; EM, embryo; ELS, embryo-like structure; MG, megagametophyte. Scale bars: 100 μm in (A), (C) and (D); 50 μm in (B).

Female gametophytes in *MSI1/msi1* mutants initiate endosperm development without fertilization and form seed-like structures that eventually abort as reported for *mea*, *fie* and *fis2*. Interestingly, the penetrance of the various *fis*-class mutants varies considerably and is much higher in *msi1* than *mea* mutants (Table III). Considering that many *fis*-class alleles are likely null alleles, the difference in penetrance could be attributed to partial redundancy of MEA and other E(Z) homologues in *Arabidopsis*. In contrast, the high penetrance in *MSI1/msi1* suggests that none of the other four MSI1-like proteins in *Arabidopsis* (MSI2–5) can substitute for MSI1 function in preventing endosperm development in the absence of fertilization. In fact, in *msi1* mutant gametophytes fertilization-independent endosperm formation occurred even when the gynoecia were pollinated, leading to the formation of a diploid endosperm surrounding the embryo. This suggests that the need for double fertilization is strongly relaxed. Because embryo development was never observed without pollination, we conclude that after pollination only the egg cell is fertilized while the central cell nucleus starts to divide without fertilization. Two main hypotheses attempt to rationalize the evolutionary origin of the endosperm (Friedman, 2001; Baroux *et al*., 2002; Berger, 2003). One hypothesis assumes a sporophytic nature of the endosperm, which would have developed from an altruistic second embryo. The other hypothesis suggests that the endosperm is derived from the female gametophyte and the second fertilization event developed only later to prevent accumulation of nourishing endosperm tissue in the absence of an embryo. The unique phenotype of *MSI1/msi1* provides some support for the idea of a gametophytic endosperm origin.

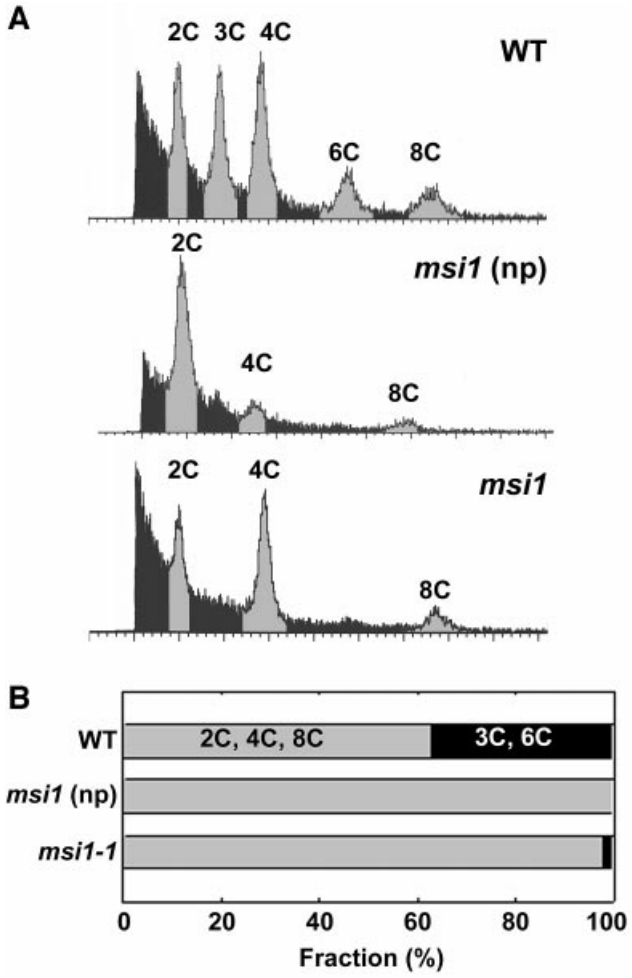


Fig. 7. Mutant *msi1* seeds contain diploid endosperm. (**A**) Ploidy analysis of nuclei from wild-type seeds at 6 DAP (WT), *msi1* seeds developing without fertilization (*msi1*, np) and *msi1* seeds at 6 DAP (*msi1*). (**B**) Quantified results from ploidy analysis ($n \geq 4$, SD < 5%).

Our results do not explain directly why *msi1* mutant seeds contain abnormal embryos and abort. Since *MSI1 is* expressed in the developing embryo, it is most likely also functionally required during embryo development. However, it is also possible that the defective endosperm perturbs embryo development. Distinguishing between these two possibilities will be subject of future investigations. Several forward genetic screens for *fis*-class mutants have been reported in which several alleles of *mea*, *fie* and *fis2* were isolated. Considering the high penetrance of the *fis* phenotype in *MSI1/msi1* mutants, it is striking that no mutant allele of MSI1 was previously identified. It is possible that the screens were not yet saturated, suggesting that still other *fis*-class mutants can be expected.

The function of the MSI1–FIS–MEA PcG-like complex is separate from other MSI1 functions

FIE and MEA interact, suggesting that they function together in a complex similar to PcG complexes in animals. We find that the *Arabidopsis* MEA–FIE complex has a molecular mass of about 600 kDa and therefore is similar to the *Drosophila* 600 kDa ESC/E(Z) complex and the homologous complex from mammals (Ng *et al*., 2000; Tie *et al*., 2001; Czermin *et al*., 2002; Kuzmichev, 2002; Müller *et al*., 2002). MSI1 co-migrates with MEA and FIE and can be co-immunoprecipitated with both proteins *in vivo*. These results show that MSI1 is an additional

Table III. Penetrance of fertilization-independent seed development (6 DAP)

	Seed-like	Ovules	Percentage seed-like	Penetrance (%)	Reference
MSI1/MSI1	0	146	0.0	NA	This work
MSI1/msi1	147	210	41.2	82.4	This work
MEA/mea3 (Ler)			7.5	15.0	Kiyosue *et al*. (1999)
MEA/mea3 (Col)			10.3	20.6	Kiyosue *et al*. (1999)
MEA/mea4 (WS)			1.8	3.6	Kiyosue *et al*. (1999)

NA, not applicable.

member of the FIE–MEA complex. *In vitro* binding experiments revealed that MSI1 binds efficiently to FIE but not to MEA, suggesting that the topology of the FIS complex involves FIE as a bridge between MEA and MSI1. While MEA function is specific for seed development, MSI1 and FIE were also shown to act during later stages of development. In particular, MSI1 is part of chromatin assembly factor complex CAF 1 that is required for the maintenance of shoot and root apical meristems. However, *fas1* and *fas2*, which lack the two other CAF-1 subunits (Kaya *et al*., 2001), do not show seed abortion and fertilization-independent endosperm or seed development. The normal reproductive development of *fas* mutants demonstrates that MSI1 acts independently of CAF-1 in seed development. Furthermore, MSI1 is the only MSI1-like protein that is strictly required for seed development in *Arabidopsis* as mutants in any of the other four *MSI1*-like genes had normal seed set (data not shown). However, additional functions of *MSI1* could account for the more severe phenotype of *msi1* compared with *mea* or *fie* mutants.

The combined molecular mass of MEA, FIE and MSI1 is only 169 kDa, suggesting that additional proteins are present in the complex. One likely candidate is FIS2, although no direct interaction has been observed so far (unpublished results). The MEA–FIE–MSI1 complex is most likely conserved between monocots and dicots, because all members of this complex have homologues in monocots like maize (Rossi *et al*., 2001; Springer *et al*., 2002; Danilevskaya *et al*., 2003).

Together, we demonstrate the presence of a 600 kDa PcG-like complex in *Arabidopsis* that consists of MEA, FIE and MSI1. This complex is of ancient origin and has been evolutionarily conserved between plants and animals. Similar to *MEA* and *FIE*, *MSI1* is a gametophytic maternal effect gene because the paternal copy of *MSI1* has no effect on the fate of the offspring. In *msi1*, endosperm development of mutant seeds initiates independently of fertilization even in pollinated gynoecia, leading to the formation of embryos surrounded by diploid endosperm. This most likely leads to an earlier arrest of embryo development compared with *mea* and *fie* mutants. Overall, our reverse genetics approach has facilitated the identification of a new subunit of the MEA–FIE complex and revealed the role of MSI1 in the correct initiation and progress of seed development.

Materials and methods

Plant material and growth conditions

Plants were grown in Conviron growth chambers with mixed cold inflorescent and incandescent light (150 μmol/m^2/s, 23°C) under long days (16 h of light). Seeds of Columbia and Landsberg *erecta* wild-type accessions and of *fas1-1* and *fas2-1* (Reinholz, 1966; Leyser and Furner, 1992) mutants were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC On-Line Catalogue at http://nasc.nott.ac.uk/home.html).

To complement the *MSI1/msi1* mutant, a 2000 bp fragment upstream of the *MSI1* start codon was amplified by PCR using primers CGGGATCCAGGTTTGGAATCGACCAAGA and TGTCGACCGATGTCTTTGTTATTCCCG. The *MSI1* cDNA was amplified using primers CGGTCGACATGGGGAAAGACGAAGAGGAA and GACCATGGAAAGAAGCTTTTGATGGTTCTTC, and *MSI1* promoter and cDNA were inserted into a modified pCAMBIA1380 vector. Heterozygous *msi1* plants were transformed by floral dip and transgenic plants were selected on hygromycin. T1 plants were assayed for complementation of seed abortion and several complementing lines were obtained. After performing DNA blots with an *HPT* probe, we chose a line with a single insert for further analysis (*msi1 compl.-1*)

RNA isolation and RT-PCR

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For RT-PCR analysis, 1 μg total RNA was treated with DNase I. Half of the DNA-free RNA (0.5 μg) was reverse transcribed using an oligodT primer and MMLV reverse transcriptase (Clontech, Palo Alto, CA), while the remaining RNA was incubated without reverse transcriptase. Aliquots of the generated cDNA, which equalled 50 ng total RNA, were used as template for PCR with gene-specific primers AtMSI1-F (GCACCGCTCTTCACACATTTG) and AtMSI1-R (TCGATCCTGCTAAGGTCCCAA) or GAPDH-F (TTCTTGGCACCAGCTTCAAT) and GAPDH-R (CTCCCTTGGAAGGAGCTAGG).

In situ hybridization

In situ hybridization was performed as described (Vielle-Calzada *et al*., 1999) with modifications. For sense and antisense 11-digoxigenin-UTP-labelled probes, a plasmid pKS (Stratagene) containing a 450 bp fragment (5′ end of the *MSI1* cDNA) was linearized with restriction enzymes cutting in the polylinker (*BamH*I and *Hind*III, respectively) and 1 μg was used for probe synthesis. Mature flowers and siliques of *Arabidopsis thaliana* wild-type plants were fixed in 4% paraformaldehyde and embedded in Paraplast Plus (Sigma). Sections 10 μm thick were cut using a Leica microtome (Leica RM 2145) and mounted on ProbeOnPlus slides (Fischer Biotech). Sections were digested with proteinase K for 30 min at 37°C, treated with acetic acid anhydride, dried in ethanol and then hybridized with about 40 ng of DIG-labelled probes per slide overnight at 55°C. After washing with 0.2× SSC at 55°C, the slides were processed for detection of the DIG antigen. This involved blocking with DIG-blocking reagent and bovine serum albumin, followed by incubation with an anti-DIG antibody conjugated to alkaline phosphatase (Roche). After washing with blocking reagent, the immunological detection was performed by incubation in NBT and X-phosphate for periods of 16 to 18 h. Reactions were stopped with TE buffer 10 mM (pH 8.0) and mounted in TE/glycerol (v/v) for microscope analysis.

Histological analysis

Material was fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) overnight at 4°C and embedded in Technovit 7100 resin (Kulzer, Wahrheim, Germany). Sections 5 μm thick were stained with Toluidine blue and observed using an Axioplan 2 microscope (Zeiss, Jena, Germany). Alternatively, tissues were cleared with chloralhydrate after fixation in ethanol–acetic acid (9:1) and observed under differential interference contrast (DIC) optics. Images were recorded with an Axiocam HRC CCD camera (Zeiss, Jena, Germany) and edited with ZeissVision software.

Gel filtration analysis

Inflorescences (3 g) were harvested and immersed in 30 ml of Buffer 1 [1 M hexylene glycol, 10 mM PIPES pH 7.0, 10 mM $MgCl_2$, 0.2% Triton X100, 5 mM β-mercaptoethanol, 1 μM $ZnSO_4$, 0.8 mM phenylmethylsulfonyl fluoride (PMSF), 1× complete Protease Inhibitors (PI) (Roche)]. The tissue was homogenized using a Polytron and then filtered through 500 μm and 50 μm meshes. The solution was centrifuged (2000g) for 10 min and the pellet was resuspended in 15 ml of Buffer 2 (0.5 M hexylene glycol, 10 mM PIPES pH 7.0, 10 mM $MgCl_2$, 0.2% Triton X100, 5 mM β-mercaptoethanol, 0.8 mM PMSF, 1× PI). After centrifugation (3000g) for 10 min, the pellet was resuspended in 500 μl of Buffer 3 (110 mM KCl, 15 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 μM $ZnSO_4$, 1 mM dithiothreitol, 1× PI). Then, 50 μl of 4 M ammonium sulfate solution was added slowly and the solution was rocked on ice for 30 min. The solution was dialysed against NLB, and dialysed extracts (200 μl) were loaded onto a 14 ml Bio-SEC-250 column (Bio-Rad) and eluted with Buffer 3 at a flow rate of 0.5 ml/min. Fractions (200 μl) were collected and precipitated with acetone. The precipitate was resuspended in SDS sample buffer and analyzed on protein blots using anti-MEA, anti-FIE (Köhler *et al*., 2003) and anti-MSI1 (Ach *et al*., 1997; Hennig *et al*., 2003) antisera.

Immunoprecipitations

Anti-MEA and anti-FIE antibodies were cross-linked to protein A agarose as described previously (Weigel and Glazebrook, 2002). Nuclei were isolated as described above and washed with Buffer 2. Nuclei were dissolved in 300 μl of Buffer 4 (50 mM Tris pH 7.5, 10 mM EDTA, 1%

SDS, 1× PI) and the extract was diluted 10-fold with Buffer 5 (1.1% Triton X100, 1.2 mM EDTA, 16.7 mM Tris pH 7.5, 167 mM NaCl, 1× PI). Extracts were precleared for 1 h with protein A beads. Antibodies coupled to protein A beads (20 µl) were added to 700 µl nuclear extract and incubated under rotation for 4 h at 4°C. Immunocomplexes were washed four times with 1 ml of Buffer 6 (10 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X100, 0.5% NP 40, 1× PI) and eluted by boiling with sample buffer.

In vitro pulldown assays

The full-length *MSI1* coding sequence was cloned into the pGEX-4T expression vector (Amersham). *Escherischia coli* strains harbouring the pGEX-MSI1 or pGEX plasmids were grown overnight in 2× YT medium at 37°C. Cultures were diluted 1:100 into fresh medium and grown until OD_{600} = 0.8 followed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. After induction for 6 h at 28°C the cells were pelleted and resuspended in BB (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X100, 1 µM ZnSO4, 1 mM PMSF, 1× PI). Lysozyme was added to 2 mg/ml and cells were lysed by incubation for 20 min on ice. After a brief sonication the solution was centrifuged (20 000*g*) for 10 min, and aliquots of the supernatant were frozen in liquid nitrogen. Expression of FIE protein using the pRSET-FIE plasmid was as previously described (Spillane *et al*., 2000). pASK-MEA contains the full-length *MEA* cDNA in pASK-IBA3 (Institut für Bioanalytik GmbH). Bacterial cells containing pASK-MEA were grown under similar conditions to those described for pGEX-MSI1. Expression was induced by adding anhydrotetracyclin to 0.2 µg/ml. After 6 h of induction, cells were pelleted and lysed as described above. Equal volumes of extract containing FIE or MEA were combined with extracts containing GST-MSI1 or GST and incubated under rotation for 2 h. Then, 100 µl of glutathione beads were added and incubation continued for 40 min. After six washes with BB, bound proteins were eluted with SDS sample buffer and analyzed on protein blots using anti-Xpress antibodies (Invitrogen) for detection of Xpress-FIE or anti-MEA antibodies.

Ploidy analysis

Ploidy analysis was performed as described (Matzk *et al*., 2000). About 50 seeds were isolated from siliques, crushed with a pestle in microfuge tubes containing 400 µl nuclear extraction buffer (Partec, Münster, Germany), incubated for 30 min on ice, filtered through 30 µm mesh, mixed with 1 ml nuclear staining buffer (Partec), incubated on ice for a further 10 min and analyzed with a Partec Ploidy Analyser. For quantification, results of four to six independent preparations were averaged.

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