Identification of new members of Fertilisation Independent Seed Polycomb Group pathway involved in the control of seed development in Arabidopsis thaliana

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Summary

In higher plants, double fertilisation initiates seed development. One sperm cell fuses with the egg cell and gives rise to the embryo, the second sperm cell fuses with the central cell and gives rise to the endosperm. The endosperm develops as a syncytium with the gradual organisation of domains along an anteroposterior axis defined by the position of the embryo at the anterior pole and by the attachment to the placenta at the posterior pole. We report that ontogenesis of the posterior pole in Arabidopsis thaliana involves oriented migration of nuclei in the syncytium. We show that this migration is impaired in mutants of the three founding members of the FERTILIZATION INDEPENDENT SEED (FIS) class, MEDEA (MEA), FIS2 and FERTILIZATION INDEPENDENT ENDOSPERM (FIE). A screen based on a green fluorescent protein (GFP) reporter line allowed us to identify two new loci in the FIS pathway, medicis and borgia. We have cloned the MEDICIS gene and show that it encodes the Arabidopsis homologue of the yeast WD40 domain protein MULTICOPY SUPPRESSOR OF IRA (MSI1). The mutations at the new fis loci cause the same cellular defects in endosperm development as other fis mutations, including parthenogenetic development, absence of cellularisation, ectopic development of posterior structures and overexpression of the GFP marker.

Movies and supplemental data available online

Key words: Arabidopsis thaliana, Endosperm, Seed, FIS, Polycomb, MSI1

Introduction

In the flowering plant Arabidopsis thaliana, double fertilisation initiates seed development (reviewed by Raghavan, 2003). One sperm cell fertilises the egg cell and initiates embryogenesis and a second sperm cell fertilises the central cell that develops into the endosperm. Endosperm development initiates as a syncytium. The endosperm is part of the seed and is thought to play an essential role in the control of maternal nutrient fluxes to the embryo. After four cycles of synchronous syncytial divisions, three mitotic domains are established along the anteroposterior axis of the endosperm and define the anterior micropylar, the peripheral and the posterior chalazal domains (Boisnard-Lorig et al., 2001). At the eighth mitotic cycle, cellularisation of the syncytial endosperm is initiated in the anterior domain around the embryo, prior to cellularisation of the peripheral domain (Sørensen et al., 2002). In contrast, the posterior endosperm does not cellularise and consists of multinucleate masses of cytoplasm, defined as the cyst at the most posterior location, and as nodules when located at the anterior part of the cyst (Scott et al., 1998). The organisation of a specialised posterior pole has been widely conserved through evolution (Floyd and Friedman, 2000). In Arabidopsis, mitotic division has not been observed in the posterior domain and the origin of the several nuclei present in this domain has remained unclear.

In Arabidopsis, the genes MEDEA (MEA) and FERTILIZATION INDEPENDENT SEED 2 (FIS2) encode the Polycomb group (PcG) protein homologues of Enhancer of zeste (E(Z)) and Suppressor of zeste 12 (SU(Z)12) in Drosophila, respectively (Grossniklaus et al., 1998; Luo et al., 1999). MEA interacts in a PcG complex with the Arabidopsis homologue of Extra Sex Combs (ESC), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) (Ohad et al., 1999; Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000) and FIS2 is likely to be a third member of this PcG complex (Berger and Gaudin, 2003; Köhler et al., 2003a; Reyes and Grossniklaus, 2003). The fis mutants were originally isolated for the capacity to initiate seed development in absence of fertilisation (Peacock et al., 1995; Ohad et al., 1996; Chaudhury et al., 1997). Autonomous seeds do not contain an embryo but only endosperm.

Another original feature shared by fis mutants is a gametophytic maternal effect on seed abortion. The FIS class gene MEA was originally identified in a screen for female
gametophytic mutants affecting embryo sac development and function or displaying maternal effects (Grossniklaus et al., 1998). Seeds derived from female gametophytes carrying a mutation in one of the FIS genes abort irrespective of whether the paternal allele is mutant or wild type (WT). Among other phenotypes, the fis class mutants for FIE, MEA and FIS2 are all characterised by an abnormal development of the endosperm posterior pole with a cyst and nodules larger than in the WT and the ectopic location of nodules in the peripheral endosperm (Sørensen et al., 2001). The endosperm of fis mutants shares other common features such as the absence of cellularisation and overproliferation at late stages (Kiyosue et al., 1999; Vinkenoog et al., 2000; Sørensen et al., 2001).

We have previously isolated the enhancer trap green fluorescent protein (GFP) marker line KS117 that displays uniform GFP expression in the endosperm until the embryo dermagon stage and later becomes confined to the posterior pole (Haseloff, 1999) (http://www.plantsci.cam.ac.uk/Haseloff). In contrast to the WT, KS117 GFP expression in a fis mutant background is uniform throughout endosperm development and dramatically over-expressed as early as the embryo octant stage (Sørensen et al., 2001).

In this study, we took advantage of KS117 GFP reporter gene expression to compare the dynamics of endosperm posterior pole formation in the WT and fis mutants. We screened for altered KS117 GFP expression to identify mutants that showed defects in endosperm patterning pertaining to the posterior pole. We isolated two new members of the fis class, medicis and borgia. MEDICIS encodes the WD40 domain protein M511 that has recently been demonstrated to directly interact with the FIS class protein FIE in the MEA/FIE PcG complex (Köhler et al., 2003a). Although medicis and borgia display autonomous endosperm development as do other fis mutants, they show distinctive genetic and phenotypic features.

Materials and methods

Plant material and growth conditions

The KS117 enhancer-trap line was generated in J. Haseloff’s lab (www.plantsci.cam.ac.uk/Haseloff/home.html) and was described previously (Sørensen et al., 2001). fis1/mea, fis2-3, fis3/fie lines were provided by A. Chaudhury (Chaudhury et al., 1997). METI a/s line was provided by J. Finnegan (Finnegan et al., 1996). Plants were grown at 20°C in a growth chamber with a 12-hour day/12-hour night cycle until they formed rosettes. Flowering was then induced at 22°C with a 16-hour day/8-hour night cycle in a greenhouse. Autonomous seed development was obtained after emasculation of mature flower buds under greenhouse conditions. Pistils were harvested 7 days after emasculation.

Microscopy and image processing

Developing seeds were isolated from individual siliques at different stages of development. Each population of seed was mounted in Hoyer’s medium (Boisnard-Lorig et al., 2001) and fluorescence microscopy was used for analysis of the WT and from plants heterozygous for fis1/mea, fis2-3 and fie-10 and homozygous for KS117 for analysis of KS117 reporter gene expression in fis mutants backgrounds. Freshly isolated siliques were prepared as described previously in order to be able to perform time-lapse analysis of NCDs migration (Boisnard-Lorig et al., 2001). Seeds oriented in such a way that the endosperm posterior pole was included in the confocal plane were selected for examination. Sections with 1024×1024 pixels were typically recorded every 10 minutes for at least 12 hours using a ×20 (n. a. 0.4) Ph2 Achroplan objective (Carl Zeiss, Jena, Germany) and a LSM510 Zeiss confocal laser scanning microscope. AVI films were mounted using the software Metamorph.

Genetic screening

4000 M1 plants were grown from KS117/KS117 gamma-ray irradiated seeds (200 gray at a rate of 27 gray/minute) and the main stem cut to allow development of lateral sectors. One sector was chosen per plant and one siliquae was slit open and seeds examined at the green embryo stage. The presence of 10% semi-stereile mutations (absence of development of 50% of seeds), 8% embryo lethal mutation (collapsed seeds) and 1.1% albino mutations (seeds with a white embryo) was recorded. These percentages compared with those obtained in EMS screens (Jürgens et al., 1991) allowed us to estimate the expected allelic frequency of the screen to be one to two alleles per mutation. In parallel, we mounted a small population of seeds isolated from one or two siliques in mounting medium on a microscope slide. Mounting medium consisted of 0.3% plant agar (Duchefa, The Netherlands) in Murashige and Skoog culture medium (Sigma, Saint-Quentin Fallavier, France). Seeds were isolated at the embryonic heart stage when KS117 expression is confined to the posterior pole in the WT. We observed GFP fluorescence patterns of the KS117 marker using a Leica MZFLIII stereomicroscope equipped with a ×1.6 planApo objective (Leica, Jena, Germany) coupled to a DC3000 digital camera (Leica Microsystems, Heerburg, Germany). Images were processed with the FW4000 software (Leica). Four backcrosses to WT KS117/KS117 were performed for each line.

Genetic mapping

F2 mapping populations were generated by crossing each mutant line to WT Columbia. Putative alleles of mea, fis2, fie and dme were identified through a low recombination rate with the following markers, respectively: nT7123 (for primer sequences, see the TAIR database (www.arabidopsis.org), fis2sslp (5'-AATTGAGCCCTCTT-GACGT TTGTGTA-3' and 5'-CTCGAATTTTGGCTAGTA-GAA-3'), CER45684 [designed from Cereon database (www.arabidopsis.org/Cereon/index.html) (Jander et al., 2002), 5'-AACC CTAAGTGCTAGTTATGC-3' and 5'-CCAAGCTAGTAA-TGCACAATGAGAAC-3] and CER47946 (Cereon, 5'-GAGTCGAGCCTTAGATAGACGC-3' and 5'-CTGTTGGTCTACGTGGCATCAAG-3'). Allelism to mea, fie or dme was confirmed by direct
sequencing of the gene in the mutant line and comparison to the WT sequence. Allelism to fis2 was confirmed by phenotypic complementation. The putative fis2 alleles JF2034 and JF2206 were crossed as homozygotes with a line containing the 18H1 cosmid containing a wild-type copy of FIS2 linked to kanamycin resistance (KanR) (Luo et al., 1999). F1 plants were heterozygous for the mutation and hemizygous for 18H1. In F1 plants, half the mutant ovules carry a WT copy of FIS2 provided by 18H1 cosmid. Thus, if complementation takes place, only 25% of seeds are expected to display the mutant phenotype in contrast to a selfed heterozygote complementation takes place, only 25% of seeds are expected to produce 50% mutant seeds. Four independent KanR F1 plants were analysed for each line and showed 25% mutant seeds instead of the 50% observed in heterozygous plants [JF2034×18H1 no. 1: 23.7% mutant seeds (s.d. 6.5); no. 2: 27.3% (4.5); no. 3: 25.2% (5.5); no. 4: 24.2% (4.6). JF2206×18H1 no. 1: 24.7% (4.3); no. 2: 21.7% (3.2); no. 3: 24.4% (5.5); no. 4: 22.5% (2.6)]. The locus associated with the bga mutation was located between TAIR markers PLS7 and nga1126. MEDICIS was shown to be located between MTI20.1/2 (Cereon, 5′-AACCCTTCTTTTATCATTCTTC-3′ and 5′-TCAGTACCTACCTACGAAAGTC-3′) and 1194/22.4/5 (Cereon, 5′-AGTTTTCGAAGCCAGGCTGAT-3′ and 5′-CCAAACGGGATGTAATCATCTGTT-3′).

Results

Ontogeny of endosperm posterior pole involves oriented migrations of nuclei

Until the embryo dermatogen stage when the syncytial endosperm has undergone six mitotic divisions and contains approximately 50 nuclei, the posterior pole contains two or four nuclei that do not divide but become larger as a result of endoreduplication cycles (Boisnard-Lorig et al., 2001) (Fig. 1A). These large nuclei are at the centre of a mass of cytoplasm called the cyst and are surrounded by nuclei embedded in small masses of cytoplasm forming nuclear cytoplasmic domains (NCDs; n=22) (Brown et al., 1999). After the seventh cycle of syncytial nuclear division in the endosperm (early globular stage embryo) the cyst still contains two or four large nuclei and is surrounded by one or two NCDs containing two nuclei (Fig. 1B) (n=18). Until the next mitotic division that is followed by endosperm cellularisation (early heart stage embryo) four to seven NCDs with multiple nuclei are observed above the cyst. These structures, called nodules, contain up to ten nuclei (Fig. 1C) (n=68). In the cyst the large nuclei are surrounded by ten to 30 nuclei similar in size to the nuclei in NCDs (n=68). The origin of multinucleate nodules and the cyst is unknown. It could be explained either by divisions of nuclei within NCDs or by fusion of NCDs together or with the cyst. To distinguish between these two mechanisms, we performed time-lapse recording of endosperm posterior pole development (Fig. 2A; movie 1, http://dev.biologists.org/supplemental/). In the enhancer trap line KS22, NCDs are labelled with mGFP5 targeted to the endoplasmic reticulum and endosperm develops as in the WT (Boisnard-Lorig et al., 2001). Recordings were made after the seventh cycle of syncytial mitosis and show that this event is followed by migration of NCDs toward the cyst (Fig. 2A, 170 minutes) (n=10). We observed that a few NCDs fuse into the cyst (movie 1, http://dev.biologists.org/supplemental/). More frequently a NCD migrates toward another NCD located closer to the cyst and merges with it. The fusion of two NCDs constitutes a multinucleate nodule (Fig. 2A, 370-430 minutes). Nodules incorporate other NCDs (Fig. 2A, 590-660 minutes) and eventually migrate and merge with the cyst (movie 1, http://dev.biologists.org/supplemental/). Migration of NCDs takes place within 30-60 minutes for relatively short distances (30-60 m) along the anteroposterior (AP) axis is indicated by the green double arrow. (A) Embryo dermatogen stage, endosperm stage VII with approx. 50 nuclei. Two large nuclei in the cyst have undergone two cycles of endoreduplication and are embedded in a pool of cytoplasm. Other nuclei in the peripheral domain are surrounded by a small mass of cytoplasm that constitutes a NCD. Scale bar: 25 μm. The 3D reconstruction was obtained from 85 optical sections of 0.4 μm thickness. (B) Early globular stage embryo, endosperm stage VIIa with approx. 100 nuclei. Above the cyst that still contains only two large nuclei, are two masses of cytoplasm that each contain two nuclei (arrows). Other NCDs are still attached to the walls of the peripheral endosperm. Scale bar: 25 μm. The 3D reconstruction was obtained from 46 optical sections of 0.65 μm thickness. (C) Early heart stage embryo, endosperm stage IX with cellularisation initiated in the micropylar endosperm at the anterior pole (not visible). The 3D reconstruction is represented as an open book split in the middle, along a plane aligned with the anteroposterior axis. The cyst contains multiple nuclei of similar size to those in NCDs. Above the cyst, are observed seven nodules that contain from two up to ten nuclei. In more anterior domains, free NCDs line the wall of the peripheral endosperm. Scale bar: 50 μm. The 3D reconstruction was obtained from 96 optical sections of 0.4 μm thickness.
syncytial mitotic divisions and includes 25-30 nuclei after the eighth mitotic division. This division is followed by endosperm cellularisation. The posterior pole does not become cellular and nuclei in NCDs still undergo massive fusion with very large nodules. Nodules and cysts show a chromatin organisation similar to nuclei in the peripheral endosperm. Scale bars: 20 μm (all sections).

Ontogeny of the endosperm posterior pole is disrupted in fis mutants

Previously, we have shown that mutations in the fis genes mea, fis2 or fie, cause ectopic KS117 GFP expression in the endosperm of the marker line, and ectopic development of nodules (Sørensen et al., 2001) (Fig. 3B). This observation led to the hypothesis that endosperm anteroposterior polarity is perturbed in fis mutants. In this study, we analysed the origin of the ectopic nodules in vivo. In fis2/FIS2; KS117/KS117 plants, strong and uniform GFP expression identifies fis2 seeds at the early globular embryo stage (Fig. 2B; movie 2, http://dev.biologists.org/supplemental/). In contrast to the WT, NCDs dynamics is strongly reduced and only limited migration is observed after the seventh mitotic syncytial division (movie 2, http://dev.biologists.org/supplemental/) (n=8). In a few cases, NCD migrations take place at random (not shown). The cyst does not appear to incorporate nodules (Fig. 2B) or undergo massive fusion with very large nodules. Nodules appear to assemble rather as the result of the growth of the cytoplasm that gradually engulfs a larger population of nuclei (Fig. 2B, Fig. 3B). Similar defects in posterior endosperm development were observed in mea and in fie backgrounds (not shown). In conclusion, fis mutations disrupt the oriented migration of NCDs and prevent proper differentiation of the endosperm posterior pole.

Endosperm posterior pole formation is maternally controlled by a group of at least six loci associated with gametophytic mutations

To identify new members of the FIS pathway, we screened a population of developing M2 seeds of 4000 M1 plants for abnormal GFP expression from the posterior endosperm marker KS117. We identified ten putatively gametophytic mutants. As heterozygotes they produced 28-50% seeds that over-expressed KS117 GFP and did not restrict its expression.
to the posterior pole (Fig. 4A-G). At the WT mature green embryo stage, mutant seeds are distinguished by a white translucent colour with a small green embryo (Fig. 4H-N). Eventually seed integuments collapse and shrivel around the embryos that arrest development at various stages after the heart stage. With the exception of JF0122, JF1762 and JF2973, plants homozygous for the mutation could be recovered from all other lines, showing that most of these mutations were not fully penetrant for seed lethality.

Production of more than 25% abnormal seeds by heterozygous plants suggested that we had isolated mutations with a gametophytic maternal control. To test this hypothesis, we reciprocally crossed each heterozygous mutant to WT. The seed-defective phenotype was only observed in the F1 when the female was the mutant (Table 1). Together, these results suggest that in these ten mutants the phenotype was under gametophytic maternal control. A rate of 50% abortion was not reached in all mutants, showing differences in penetrance.

We first determined whether these ten mutants were new alleles of known fis class genes or were new loci. Allelism to known fis mutants was shown by genetic mapping (Table 1) and was then confirmed by complementation by a WT copy of the gene or by sequencing (Fig. S1, http://dev.biologists.org/supplemental/).

During the course of this work the mutant demeter (dme) was isolated and reported to be defective in transcriptional activation of MEA (Choi et al., 2002). DME is located on chromosome 5, close to the position we determined for JF1348, suggesting that JF1348 was allelic to dme. We sequenced DME in this mutant and could confirm the allelism (Fig. S1, http://dev.biologists.org/supplemental/).

For two lines that could be mapped to chromosome 2 and 5, respectively, JF1728 and JF2973, no tight linkage to any of the known FIS genes was detected, suggesting that these two mutants affected unknown genes. We named these two mutants borgia and medicis, respectively, as a reference to the Italian families of the Renaissance period who were particularly remarkable for a tradition of infanticide as was Medea in antique Greece. The borgia mutation is located in proximity of FIS2 on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2, confirming that it is located on chromosome 2 but bga location could be narrowed down to a region that does not contain FIS2.
we had identified a new fis class locus on chromosome 2. We attribute the gene symbol BGA to this locus. Map-based cloning of medicis narrowed down the location of the mutation to a 280 kb interval that included the BACs MTI20, K21L19, MCK7 and MQ2 on chromosome 5. This portion of the Arabidopsis genome contains the MSII gene which was a candidate for the mutation. An orthologue of MSII in Drosophila, p55, is part of the PcG complex formed by genes homologous to the FIS genes MEA, FIS2 and FIE (Tie et al., 2001). In the course of this work, we learned that a T-DNA insertion in MSII was associated with a loss-of-function that caused a phenotype similar to the fis phenotype (Köhler et al., 2003a). Thus, we sequenced MSII in medicis and identified a mutation leading to the production of a very short truncated protein (Fig. S1, http://dev.biologists.org/supplemental/). We hereafter refer to the medicis mutation as msii-2.

In summary, the ten gametophytic mutants recovered from our screen identified three new mea alleles (hereafter referred to as mea-5 to mea-7), two new fis2 alleles (hereafter referred to as fis2-6 and fis2-7), two new fie alleles (hereafter referred to as fie-10 and fie-11), and one allele each of dme (hereafter referred to as dme-4) and msii1 (hereafter referred to as msii1-2). Importantly, our screen reveals the mutation bga-1 in a new fis class locus on chromosome 2.

**Effects of bga on seed development**

Taking advantage of the differential KS117 GFP expression pattern between WT and bga seeds, we established a precise description of mutant seeds development. Mutant bga embryo development is similar to the WT until early heart stage (Fig. 5A,D). From the WT mid heart stage, mutant embryo development slows down (Fig. 5B,E) and eventually arrests between the late heart and the late torpedo stages when the WT embryos reach the mature green stage. Overall WT and mutant development of endosperm are similar until the endosperm development of endosperm are similar until the endosperm contains 200 nuclei. At this stage, the WT endosperm cellularises, whereas the bga endosperm does not (n=114, Fig. 5C,F). Furthermore, an overgrowth of nodules and cyst is visible from this stage in the mutant endosperm and is amplified later in development (Fig. 6A,B). Seed size measurements show that bga and WT endosperms are similar in size, as it is the case for fie-11 at this point of seed development (Table 2). Cellularisation is followed in the WT by one cycle of pseudo-synchronous cell division leading to an endosperm containing 400 nuclei. Only half of the mutant bga syncytial endosperms undergo this division (n=39), and endosperm nuclear proliferation is arrested in bga seeds after this stage.

 Unlike other fis mutants, the bga phenotype affects only 28% seeds in siliques of bga/BGA plants fertilised by WT pollen (Table 1). No mutant phenotype was observed in seeds produced by reciprocal crosses, showing that bga mutation has a strict gametophytic maternal effect. Plants homozygous for bga are produced from seeds selected for a shrunken morphology. Transmission data confirm a high viability of the mutant embryos. When a bga/BGA plant is pollinated by BGA/BGA pollen, 21.5% (n=135 F1 plants) bga/BGA plants are found in the F1 progeny. In the reciprocal cross, fertilisation of a BGA/BGA plant by bga/BGA pollen gives rise to 45.5% (n=132) bga/BGA F1 plants. Thus, the bga mutation is fully transmitted by male gametes, whereas transmission via the female gametes is reduced by half.

In conclusion, the bga mutation has a gametophytic maternal effect on embryo and endosperm development. The embryo is first delayed from the early heart stage and then arrested at late stages. Ectopic nodule formation occurs in bga endosperm as in other fis mutants, but in contrast to what was described previously for mea and for fie (Kiyosue et al., 1999; Vinkenoog et al., 2000), no overproliferation of endosperm nuclei was observed in bga.

**msi1 has both sporophytic and gametophytic effects on seed development**

When msi1/MSII pistils are pollinated by WT plants, siliques contain 52% seeds showing a fis phenotype, while reciprocal...
New PcG mutants in *Arabidopsis*crosses produce only seeds with a WT phenotype. These results show that the *msi1 fis*-like phenotype is under gametophytic maternal control. We were not able to obtain plants homozygous for *msi1* as a result of embryo lethality. Furthermore, transmission of the *msi1* allele via the female gametes is null (*n*=232 F1 plants). Paternal transmission is also reduced in the *msi1* mutant, as only 36.2% F1 plants (*n*=232) from pollination of WT pistil by *msi1/MSI1* bear the mutant allele. Mature pollen grains from *msi1/MSI1* plants contain two gametes and present a normal morphology (not shown) and the origin of reduced paternal transmission remains unknown.

When *msi1/MSI1* plants are self-pollinated only 30% of the seeds have a *fis* phenotype and 22% of the seeds display a distinct phenotype with severe embryo abnormalities. The distribution of the *fis* and abnormal embryo phenotypes in self-pollinated plants is 2 WT:1 *fis*-like:1 abnormal embryo (*n*=157, $\chi^2=2.22 < \chi^2_{0.05(2)}=5.991$), suggesting that the abnormal embryo phenotype is under sporophytic recessive control.

KS117 GFP overexpression in mutant seeds allowed us to examine endosperm and embryo development in *msi1* self-pollinated plants. The sporophytic recessive phenotype in embryos is distinguished as early as the WT octant stage. Improper cell division patterns are observed in the embryo proper and in the suspensor (Fig. 7A,G), leading to the development of a highly abnormal embryo (Fig. 7B,H). Endosperm that surrounds the arrested embryo does not differentiate a posterior chalazal pole nor cellularise, and nucleoli are variable in size (Fig. 7C,I). In the WT, syncytial endosperm development consists of synchronous nuclear divisions. In the mutant endosperm, we observed a delay of one cycle of division at each developmental stage, in comparison to WT seeds.

The phenotype associated with the gametophytic maternal effect is distinct from the sporophytic recessive effect. During the first steps of development, the pattern and timing of division of the mutant embryos are similar to those of the WT (*n*=196, Fig. 7A,D). When WT embryos reach the mid heart stage, mutant embryos are arrested at the early heart stage (*n*=103, Fig. 7B,E). Later in development, 7% mutant embryos (n=57) develop abnormally with extra layers of cells resulting from additional periclinal divisions (not shown), as described previously in *msi1-1* allele (Köhler et al., 2003a). In the

### Table 2. Seed size and number of endosperm nuclei in *fie, bga* and *msi1* mutant seeds, at a stage corresponding to wild-type torpedo stage

<table>
<thead>
<tr>
<th>Seed genotype</th>
<th>Seed length (µm) (s.d.)</th>
<th>Student’s <em>t</em>-test</th>
<th>Seed width (µm) (s.d.)</th>
<th>Student’s <em>t</em>-test</th>
<th>No. endosperm nuclei per seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>283 (33) <em>n</em>=104</td>
<td></td>
<td>249 (33) <em>n</em>=104</td>
<td></td>
<td>400 (Scott et al., 1998)</td>
</tr>
<tr>
<td><em>fie</em>-11/FIE</td>
<td>274 (37) <em>n</em>=47</td>
<td><em>t</em>=1.49</td>
<td>246 (36) <em>n</em>=47</td>
<td><em>t</em>=0.50</td>
<td>&gt; 400 (Kiyosue et al., 1999; Vinkenoog et al., 2000)</td>
</tr>
<tr>
<td><em>bga</em>-1/BGA</td>
<td>282 (62) <em>n</em>=21</td>
<td><em>t</em>=0.11</td>
<td>241 (48) <em>n</em>=21</td>
<td><em>t</em>=0.93</td>
<td>200 (79%) 400 (21%) <em>n</em>=86</td>
</tr>
<tr>
<td><em>msi1</em>-2/MSI1</td>
<td>254 (33) <em>n</em>=69</td>
<td><em>t</em>=5.66</td>
<td>223 (32) <em>n</em>=69</td>
<td><em>t</em>=5.14</td>
<td>200 (49%) 400 (51%) <em>n</em>=39</td>
</tr>
</tbody>
</table>

Mean length and width of WT and mutant seeds are given in µm. A Student’s *t*-test was carried out on the mean length or width in comparison to WT for each mutant. *t*-values should be compared to the theoretical value of 1.96 (*α*=0.05). The number of nuclei contained in endosperm was determined after clearing of seeds at the same stage.

![Fig. 7. Microscopic analysis of *msi1*-2/MSI1 and *msi1*-2/*msi1*-2 seeds. (A,B) WT embryo at dermatogen and mid heart stage, respectively. em: embryo proper, su: suspensor. (C) Cellularised endosperm in a WT seed at the same stage as in B. (D,E) *msi1*-2/MSI1 embryos in same siliques as A and B, respectively. (F) Endosperm is not cellularised in a *msi1*-2/MSI1 seed at the same stage as in C. (G,H) *msi1*-2/MSI1-2 embryos in same siliques as A and B, respectively. (I) Endosperm is not cellularised in a *msi1*-2/*msi1*-2 seed at the same stage as in C. Nucleoli are variable in size. Scale bars: 50 µm.](image-url)
endosperm, the overall pattern and the pace of nuclei proliferation are not distinguishable from the WT until the eighth cycle of mitosis takes place. At this stage, the endosperm contains 200 nuclei and, whereas it cellularises in the WT, no cellularisation is observed in the mutant (Fig. 7C,F) where cyst and nodules overgrowth is detected. Later on, at the WT torpedo stage, overgrowth of nodules and a large development of the cyst are dramatic in msi1/MSI1 seeds (Fig. 6C). However, at this stage of development, endosperm in mutant seeds is smaller than in WT. This is accompanied by a reduced proliferation of endosperm nuclei in comparison with WT (Table 2).

In conclusion, the msi1-2 mutation causes a severe gametophytic maternal effect on endosperm and embryo development, and a distinct sporophytic recessive embryo lethality. The pace and pattern of cell and nuclei divisions are severely affected in msi1/msi1 embryo and endosperm, as early as the octant stage in the WT embryo. The gametophytic maternal effect causes the arrest of embryo development and affects several features of endosperm development as other fis mutations. But, unlike other mutations (Kiyosue et al., 1999; Vinkenoog et al., 2000), the msi1 gametophytic maternal phenotype includes a reduction of growth and proliferation of endosperm after the stage when WT endosperm cellularises.

**msi1 and bga mutations promote autonomous seed development**

The mutants mea, fis2 and fie are able to initiate endosperm development in the absence of fertilisation. Autonomous endosperm development is accompanied by increase in pistil elongation, which was used as a criterion to identify alleles representative of several fis class loci (Fig. 8A-G). We observed very little or no pistil elongation for dme-4 as reported for dme-l (Choi et al., 2002). In contrast, pistil elongation was marked for the other loci, with increasing strength in the following order, bga, mea, fis2, fie and msi1. Endosperm development is characterised by an increase in ovule size leading to a small developing seed that contains autonomous endosperm (Ohad et al., 1996; Chaudhury et al., 1997). We quantified the penetrance of mutations at each locus by counting the number of autonomous seeds relative to the total number of ovules that are likely to carry the mutation (Table 3, Fig. 8H-N). The penetrance of each mutation is correlated with the degree of pistil elongation. While no autonomous seed development was observed in dme/DME, 25-92% of the mutant ovules of other fis/FIS plants undergo some degree of seed development in the absence of fertilisation. The allele msi1-2 has the highest rate of autonomous seed development, with a penetrance of 92%, suggesting that almost every ovule that inherits the msi1-2 mutation undergoes autonomous seed development. We conclude that, unlike dme, which does not promote autonomous seed development, bga and msi1 represent true new members of the fis class of mutants. As observed for alterations of endosperm development and penetrance of seed abortion, bga has the weakest effects on autonomous seed development while msi1 causes the strongest defect in this trait.

**Demethylated pollen restores ectopic GFP expression in mea, fis2, dme and bga but not in fie and msi1**

It has been shown that pollination of a WT plant by a demethylated genome reduces the size of the resultant seeds (Adams et al., 2000). When fertilised by pollen from a METHYLTRANSFERASEI antisense transgenic line (MET1 a/s), in which global genome methylation is reduced to 20% of the WT level (Finnegan et al., 1996), mea, fis2 or fie ovules develop into seeds whose size and shape are close to WT (Luo et al., 2000; Vinkenoog et al., 2000). This has been interpreted as a rescue of fis phenotype by introducing a demethylated paternal genome or by the dominant effect of the MET1 a/s transgene. We observed the same result with the other gametophytic maternal mutants: dme/DME × MET1 a/s siliques contained 52.8% large seeds (n=193), bga/BGA × MET1 a/s cross produced 30.4% large seeds (n=269) and msi1/MSII × MET1 a/s showed 45.5% large seeds (n=213).

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**Table 3. The fis class mutants bga-1 and msi1-2 undergo autonomous seed development**

<table>
<thead>
<tr>
<th>Line</th>
<th>No. autonomous seeds</th>
<th>No. undeveloped ovules</th>
<th>% seeds/total ovules</th>
<th>Penetrance</th>
</tr>
</thead>
<tbody>
<tr>
<td>dme-4/DME</td>
<td>0</td>
<td>532</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mea-6/MEA</td>
<td>70</td>
<td>292</td>
<td>20.6±11.5</td>
<td>41.2</td>
</tr>
<tr>
<td>fis-2/FIS2</td>
<td>86</td>
<td>189</td>
<td>31.7±14.4</td>
<td>63.4</td>
</tr>
<tr>
<td>fie-11/FIE</td>
<td>166</td>
<td>265</td>
<td>38.5±11.4</td>
<td>77.0</td>
</tr>
<tr>
<td>bga-1/BGA</td>
<td>24</td>
<td>168</td>
<td>12.8±6.4</td>
<td>25.6</td>
</tr>
<tr>
<td>msi1-2/MSII</td>
<td>264</td>
<td>320</td>
<td>46.2±10.4</td>
<td>92.4</td>
</tr>
</tbody>
</table>

Heterozygous mutants (one from each group) were emasculated. Number of seeds (enlarged ovules) and undeveloped ovules was determined 7 days after emasculation. % seeds are the percentage of ovules that undergo autonomous development. Penetrance estimates the percentage of ovules carrying the mutation that undergo autonomous development.
Thus, fertilisation by demethylated pollen seems to rescue the visible phenotypes in the three new mutants *dme*, *bga* and *msi1*, as was described for *mea*, *fix2* and *fie*. In order to investigate whether *MET1* a/s polination was also able to rescue the GFP expression phenotype, we examined the GFP expression in siliques resulting from pollination of *fis/FIS*; KS117/KS117 plants with pollen from homozygous *MET1* a/s plants (Fig. 9). Nine days after pollination, KS117 expression was weak and restricted to the posterior pole in small seeds that had inherited the WT maternal *FIS* allele. In contrast, large seeds presented various KS117 expression patterns, depending on the *fis* locus. In *mea*, *fix2*, *dme* and *bga* seeds (Fig. 9A,B,D,E) KS117 expression was restricted to the posterior cyst, as in WT (*n=39, 194, 134 and 154, respectively). In contrast, seeds that maternally inherited the *fie* (18 seeds/56) or *msi1* (34 seeds/71) alleles presented a strong uniform KS117 expression as if they had been pollinated by WT pollen (Fig. 9C,F). These results suggest that the rescue of the gametophytic maternal effect by a demethylated male genome uses alternative pathways depending on the *FIS* gene involved.

**Discussion**  
The endosperm posterior pole development involves oriented nuclear migrations that are under the control of the MEA/FIE PcG complex  
The origin of the multiple nuclei present in nodules and in the cyst of the posterior pole endosperm was unclear since no mitosis was ever observed at this pole. Dynamic analysis showed that NCDs migrate towards the cyst and merge together to constitute multinucleate nodules. In turn, these nodules migrate and merge into the cyst that becomes multinucleate. After endosperm cellularisation, the pool of free NCDs becomes limited and remaining NCDs and nodules gradually merge into the cyst. The posterior pole ultimately consists of a large multinucleate cyst that is gradually compressed by the growing embryo after the torpedo stage. The migration of NCDs is oriented towards the posterior pole, indicating that structural features are organised in a polar fashion toward the posterior pole. The nature of these structural elements remains to be identified. Potential candidates are microtubules that have been reported to be organised in an orientation compatible with their use as tracks for migration of NCDs (Brown and Lemmon, 2001). However, the speed of migration of NCDs is at least 50 times slower than the speed recorded for migration of organelles along microtubules (Pollock et al., 1998; Carter et al., 2003). Alternatively, actin filaments may be suitable candidates, although no actin cable was reported at the posterior pole of endosperm in *Coronopus didymus*, a relative of *Arabidopsis* (Nguyen et al., 2002). Nuclear migrations have been inferred during the early endosperm development in Maize (Walbot, 1994) and in *Arabidopsis* (Mansfield and Briarty, 1990) from the analysis of fixed material. Thus, dynamic localisation of nuclei in the syncytial endosperm is a conserved feature but its mechanism remains unknown.

We had reported that *fis* mutations affect the organisation of the posterior pole with overproliferation in nodules that could be located at ectopic position (Sørensen et al., 2001). We show in this study that *fis* mutations impair the migrations of NCDs to the posterior pole. As NCDs do not migrate or migrate at random, they eventually merge at a location distant from the posterior pole and form nodules at ectopic positions. The tight regulation of NCD migration, at least in part, is under the control of the MEA/FIE PcG complex. It can be proposed that the MEA/FIE PcG complex regulates structural features, such as the cytoskeleton, involved in endosperm polarity and NCD migration. According to an alternative hypothesis, the absence of NCD migration in the *fis* mutants could reflect a general delay of endosperm development. However, such a hypothesis should involve a reduction of growth and nuclear proliferation. This is not observed during *fis* endosperm development. We therefore propose that the absence of NCD migration in *fis* mutants reveals impairment of a specific mechanism rather than a global developmental delay.

**Maternal control of development of the endosperm posterior pole depends on the conserved MEA/FIE Polycomb Group complex**  
We identified ten gametophytic maternal mutants showing a *fis* phenotype of the KS117 GFP expression pattern. Of these ten mutants, eight are allelic to *mea*, *fix2*, *fie* or *dme*. Table S1 (http://dev.biologists.org/supplemental/) compiles all the known and new mutant alleles for each of these loci. Genetic mapping identified two other loci, *bga* and *msi1*, at locations distinct from that of other known *fis* mutants. We have identified one allele for each of these two loci.

The definition of the *fis* class of mutants was based on the common ability of *mea*, *fix2* and *fie* mutants to initiate autonomous seed development. Whereas *dme* does not show this trait, the new mutants *bga* and *msi1* demonstrated autonomous endosperm development. We also show that, as described for other *fis* mutants (Kiyosue et al., 1999; Vinkenoog et al., 2000), the development of seeds that maternally inherit *bga* or *msi1* is phenotypically normal until endosperm cellularisation. From this stage, developmental defects, including perturbation of the posterior pole formation

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**Fig. 9.** KS117 GFP marker expression 9 days after pollination of heterozygous gametophytic maternal mutants by a demethylated genome. (A) *mea-6/MEA × MET1 a/s*; (B) *fix-2/FIS × MET1 a/s*; (C) *fie-1/FIE × MET1 a/s*; (D) *dme-4/DME × MET1 a/s*; (E) *bga-1/BGA × MET1 a/s*; (F) *msi1-2/MS1 × MET1 a/s*. Two types of seeds are produced when *fis/FIS* mutants are fertilised by demethylated *MET1 a/s* pollen: the small seeds (arrows) result from WT ovule development, the large seeds (arrowheads) from mutant ovules. GFP expression is low and restricted to the posterior pole in small seeds. In *mea/MEA* (A), *fix2/FIS* (B), *dme/DME* (D) and *bga/BGA* (E) seeds, KS117 GFP expression is properly restricted to the cyst, even when this structure is larger than in WT (A). In contrast, in *fie/FIE* (C) and *msi1/MS1* (F) seeds, KS117 GFP is over-expressed and not restricted to the posterior cyst (arrowheads), as when pollinated by WT. Scale bar: 200 µm (all images).
and decrease in seed viability due to embryo arrest, are observed. Ectopic nodules observed in msl1 and bga probably result from the impaired migration of NCDs although we have not performed dynamic observations in these backgrounds. Thus, the two new loci we identified on the basis of their gametophytic maternal control on endosperm development share all the typical characteristics of the fis phenotype with the exception of endosperm overproliferation at late stages, and may constitute additional members of the MEA/FIE PcG complex.

Members of the FIS class represent members of a conserved PcG complex

Our finding that medicis is mutated in the gene encoding MSI1 supports the recent demonstration of MSI1 as part of the MEA/FIE PcG protein complex (Köhler et al., 2003a). MEA, FIS2 and FIE are homologues of the Drosophila PcG proteins E(Z), SU(Z)12 and ESC, respectively that participate in a 600 kDa complex (Tie et al., 2001). This PcG complex has been analysed in detail and contains p55, the homologue of MSI1. This strongly suggests a conservation of the PcG E(Z)/ESC complex between plants and animals.

In this study we describe the new fis class mutant bga. Penetration in bga mutant is weak, suggesting that either our unique allele bga-1 is a weak allele, or the BGA protein may form a specific transient complex with the conserved MEA/FIE core complex. In Drosophila the histone deacetylase RPD3 has been shown to be part of the 600 kDa E(Z)/ESC complex in embryos (Tie et al., 2001). However, although the Arabidopsis genome contains ten members of the RPD3 family (Pandey et al., 2002), none of them is located in the vicinity of the bga locus. Alternatively, bga might be defective in a regulator of expression or imprinting of the FIS genes such as DME (Choi et al., 2002).

Interestingly, besides the embryo arrest provoked by the maternal loss of function of MSI1, we observed in msl1-2 an embryonic phenotype under a sporophytic recessive control. If the seed inherits paternal and maternal msl1-2 alleles, the embryo pattern is disrupted by cell divisions with a random orientation leading to an early arrest. Consistently abnormally enlarged nucleoli are observed in endosperm suggesting an improper control of nuclear division. MSI1 is known to be part of Arabidopsis Chromatin Assembly Factor-1 (CAF-1), together with FASCIATA1 and FASCIATA2 (Kaya et al., 2001). In vitro assays show that CAF-1 has a replication-dependent nucleosome assembly activity. Furthermore, Ach et al. (Ach et al., 1997) have shown that the tomato homologue of MSI1, LeMSI1, interacts with Retinoblastoma (Rb)-like RBB1 protein from Maize. Hennig et al. (Hennig et al., 2003) also suggest that Arabidopsis MSI1 is able to interact with Arabidopsis Rb-related RBR protein. As it is known in animals that Rb is involved in G1 phase progression (reviewed by Weinberg, 1995), it is possible that MSI1 represents a link that has already been suspected in animals between chromatin remodelling by PcG and the control of the cell cycle (Jacobs and van Lohuizen, 2002).

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