**Arabidopsis GLAUCE promotes fertilization-independent endosperm development and expression of paternally inherited alleles**

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Early seed development of sexually reproducing plants requires both maternal and paternal genomes but is prominently maternally influenced. A novel gametophytic maternal-effect mutant defective in early embryo and endosperm development, glauce (glc), has been isolated from a population of Arabidopsis Ds transposon insertion lines. The glc mutation results from a deletion at the Ds insertion site, and the molecular identity of GLC is now known. glc embryos can develop up to the globular stage in the absence of endosperm and glc central cells appear to be unfertilized. glc suppresses autonomous endosperm development observed in the fertilization-independent seed (fis) class mutants. glc is also epistatic to mea, one of the fis class mutants, in fertilized seeds, and is essential for the biparental embryonic expression of PHE1, a repressed downstream target of MEA. In addition, maternal GLC function is required for the maternal embryonic expression of the ribosome protein gene RPS5a and the AMP deaminase gene FAC1, both of which are essential for early embryo and endosperm development. These results indicate that factors derived from the female gametophyte activate a subset of the paternal genome of fertilized seeds.

**KEY WORDS:** Embryogenesis, Autonomous endosperm, Maternal effect, Paternal allele activation, Fertilization, Plant reproduction, Seed development

**INTRODUCTION**

In flowering plants, sexual reproduction involves the coordination of both male and female gametophytes and gametes for double fertilization to be realized. The new seed starts with the coordinated development of the two fertilized products—embryo and endosperm—as well as interactions between endosperm and seed coat. Very little is known about the commonalities and differences of the two fertilization events. Knowledge regarding the communication between the early embryo and endosperm is also scarce, although the early endosperm has been thought to assume a supportive and nutritive role for the early embryo (Lopes and Larkin, 1993).

The developmental programs of embryo and endosperm require both parental genomes. The extent to which each genome contributes to these two major seed components might not be equivalent, especially during early seed development before the embryo heart stage. In Arabidopsis thaliana, dominant maternal control of endosperm and embryo development has been demonstrated by the FERTILIZATION-INDEPENDENT SEED (FIS) class of genes, which includes the homologs of the genes encoding the Drosophila and mammalian Polycomb repressive complex 2 (PRC2) components MEA, FIS2, FIE, and MSI1 (reviewed by Pien and Grossniklaus, 2007). Mutations in any of these genes cause common mutant phenotypes of seeds with autonomous endosperm, abnormal cellular proliferation of fertilized embryos and endosperm, arrested heart-staged embryos, and ultimate seed abortion. Mutant phenotypes result only when the genetic lesions are present in the maternal allele inherited from the female gametophyte. In the case of MEA and FIS2, the gene products are supplied during early seed development only by the maternal alleles. Both genes are already expressed before fertilization in the embryo sac but MEA, and probably FIS2, also show imprinted maternal expression after fertilization in the developing seeds (Jullien et al., 2006b; Kinoshita et al., 1999; Luo et al., 2000; Vieille-Calzada et al., 1999). Gametophytic paternal effects on seed development have not been reported; however, preferential paternal expression in early seeds has been documented for PHERES1 (PHE1), a MADS box gene (Köhler et al., 2003b). Even in this case, only the expression of the maternal allele has been demonstrated to affect embryo and endosperm development. Maternal PHE1, a direct downstream target of maternal MEA repression, is strongly upregulated in mea seeds and partially rescues mea seed abortion when this deregulated expression is suppressed (Köhler et al., 2003b; Köhler et al., 2005).

In this study, we describe a gametophytic maternal effect mutant, glauce (glc), where the embryo develops in the absence of endosperm. In addition to affecting the fertilization of the central cell, glc genetically counters the mutants of the FIS class that control fertilization-independent endosperm formation and postfertilization embryo development. The maternally inherited glc mutant is defective in the embryonic paternal expression of PHE1 and the bi-parentally expressed genes RPS5a and FAC1, which are important for early embryo and endosperm development.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Seed sterilization, germination and seedling growth were carried out as described (Pagnussat et al., 2005). mea-8 was supplied by the ABRC stock center as line SAIL_55_B04. Transgenic FIS2::GUS, FAC1::GFP-GUS, CYCB1;1::GUS, PHE1::GUS and PIN7::PIN7::GUS seeds were gifts from Abed Chaudhury (CSIRO, Canberra, Australia), Chun-Ming Liu (Plant Research International, Wageningen, The Netherlands), Celia Baroux...
Whole-mount ovule clearing and GUS assays

These procedures were carried out as described (Pagnussat et al., 2005; Yu et al., 2005).

Confocal laser-scanning microscopy and DNA quantification of nuclei in seeds

Propidium iodide staining and confocal laser-scanning microscopy procedures were performed as described (Baroux et al., 2007) without the enzymatic treatment step, for glc/GLC seeds 1-2 days after pollination (DAP).

Pollen staining

Pollen grains from anthers of glc/GLC late-13-staged flowers (Bowman, 1994) were stained with 1 µg/ml DAPI (4',6'-diamino-2-phenylindole) in the dark for 1 hour, washed briefly in distilled water, and observed under a Zeiss Axioskop 2 microscope with both fluorescence and DIC optics.

DNA extraction, PCR and Southern blot hybridization

Genomic DNA was extracted from fresh leaves and flowers with the GenElute Plant Genomic DNA kit (Sigma, USA) or the Phytopure kit (Amersham, Switzerland) following the manufacturer's protocol. PCRs were performed with 0.5 units of Taq polymerase in 1× PCR buffer containing 1.5 mM MgCl₂, 200 µM each dNTP and 10 pmol each primer. PCR parameters were as follows: 94°C for 2 minutes, 30 cycles of 94°C/20 seconds, 52°C/30 seconds, and 72°C/1 minute, with a final extension of 72°C for 3 minutes. Thermal asymmetric interlaced (TAIL)-PCR procedures have been described previously (Parinov et al., 1999). Primers for Ds insertion site verification, cleaved amplified polymorphisms (CAPS) markers for the deletion at the Ds locus of SET2030, and for amplifying markers for the deletion at the Ds insertion site were designed.

Southern probes will be provided upon request. Southern blot analysis was performed with the DIG-Easy Hyb Kit (Roche, Switzerland) following the manufacturer's protocol or as described (Sambrook and Russell, 2001) with the DECAprime II labeling kit (Ambion, USA) and [32P]dCTP (Perkin-Elmer, USA).

Tetraploid seed generation

Meristems of glc/GLC plants undergoing the transition from vegetative to reproductive phase were treated with 0.25% colchicine. Seeds from these treated plants were pooled and germinated on MS plates containing 50 mg/l kanamycin. Seedlings were scored for resistance or sensitivity at 12 days after plating.

RESULTS

glc is a female gametophytic mutant defective in early seed development

glc was originally identified as the Ds insertion line SET2030 from a screen for female gametophytic mutants showing reduced transmission of kanamycin resistance (kanR) (Pagnussat et al., 2005). This line displayed an aberrant kanamycin resistance:kanamycin sensitivity (kanR:kanS) ratio of 0.64:1 instead of 3:1 (Table 1). Reciprocal crosses of SET2030 heterozygous plants and wild-type plants showed a severe reduction in female transmission (15%) and moderate reduction in male transmission (49%) (Table 1). To examine the cause of the female sterility, we cleared SET2030/+ siliques with Hoyer's solution (Liu and Meinke, 1998) and studied the ovule phenotype by light microscopy under Nomarski optics. Two days after early-12-staged flowers (Bowman, 1994) of SET2030/+ plants were emasculated, the embryo sacs in all ovules of the same siliques displayed wild-type morphology with four typical cells of a wild-type mature embryo sac: a central cell, an egg cell and two synergids cells (Fig. 1A). The correct cell identities of the SET2030 embryo sac were confirmed with five marker lines expressing GUS specifically in the central cell [FIS2::GUS (Luo et al., 2000) and MEA::GUS (Spillane et al., 2004)], the egg cell (ET1119 and ET1086), and the synergids (ET2634) in approximately 50% of ovules from SET2030/+ plants hemizygous for these marker lines (Fig. 1B-F).

Although SET2030 embryo sacs developed normally and established cell identity correctly before fertilization, female transmission of the Ds::kanR was significantly impaired (Table 1). Therefore, we investigated the post-fertilization phenotype of SET2030 1-3 days after pollination (DAP) in whole-mount seeds of SET2030/+ siliques. Although ~62% (n=158) of the seeds from the same siliques appeared wild-type, the remaining seeds were smaller, with embryos arrested at various stages up to the globular stage with defective endosperm (see below) and therefore were considered as mutant seeds (Fig. 2). In general, embryos of mutant seeds lagged one stage behind embryos of wild-type seeds in the same siliques. At 2.5 DAP, when most wild-type seeds of the heterozygous siliques had reached the 8- and 16-cell embryo stages, mutant seeds mainly were at the quadrant and octant stage (Table 2). Later, when wild-type seeds had reached the globular stage, most mutant seeds had collapsed, but in about 8% of mutant seeds (n=76), embryo development could proceed to the pro-globular or globular stage. Embryos of mutant seeds up to the pro-globular stage looked morphologically similar to wild-type pre-globular embryos (Fig. 2B-F), but by the late globular stage displayed some disorganization (Fig. 2G, Fig. 2I compared with Fig. 2J). Notably, in most mutant seeds, the central cell was totally devoid of endosperm development; instead, there was a single large nucleus, which could be either the unfertilized central cell nucleus or the fertilized, non-dividing primary endosperm nucleus (Fig. 2B-G, Table 2). A small proportion of mutant seeds had 2-8 endosperm nuclei of equal or unequal size (Fig. 2H, Table 2). These embryo and endosperm defects remained unchanged regardless of whether SET2030/+ flowers were pollinated with self pollen or wild-type pollen.

Since SET2030 male transmission rate was also low (Table 1), indicative of mutant effects on the male gametophyte, we tested whether the mutant male gametophyte could also be responsible for the above described phenotypes. Seeds from crosses between wild-type flowers and SET2030/+ pollen developed normally, confirming that the mutant phenotypes in the seed arise only when the mutant allele is transmitted through the female gametophyte. DAPI-stained mature pollen grains from SET2030/+ plants displayed a wild-type appearance with two sperm cells and one vegetative cell (see Fig. S1 in the supplementary material). The source of the reduced male transmission was not investigated further, but it obviously was not the cause of the observed post-fertilization seed phenotypes. We have not been able to recover SET2030/SET2030 homozygous plants from selfed SET2030/+ plants although transmission of the Ds element through both male and female germ lines occurs at low

Table 1. Transmission patterns of SET2030 (glc)

<table>
<thead>
<tr>
<th>Cross</th>
<th>kanR:kanS</th>
<th>Expected value</th>
</tr>
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<tbody>
<tr>
<td>glc+ x glc+</td>
<td>0.64 (258/405)</td>
<td>3</td>
</tr>
<tr>
<td>glc+ x wt</td>
<td>0.15 (138/907)</td>
<td>1</td>
</tr>
<tr>
<td>wt x glc+</td>
<td>0.49 (398/809)</td>
<td>1</td>
</tr>
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</table>

Raw numbers are in parentheses. The ‘expected values’ of the ratios are for the complete transmission of the Ds( kanR) allele: kanR, kanamycin resistant; kanS, kanamycin sensitive.
frequency (Table 1), implying embryo lethality of homozygous seeds. We named this mutant glauce (glc) after the mythological princess of Corinth (Euripides, 431 BC), because of its antagonistic relationship with medea (see below). We concluded that glc is a gametophytic maternal-effect mutant defective in early endosperm development and possibly in central cell fertilization, and partially affecting early embryogenesis.

The glc mutation results from a deletion at the Ds insertion site on chromosome 1
The genomic sequences flanking the Ds insertion site in glc/GLC plants were amplified by TAIL-PCR (Liu et al., 1995). We found that the 3' end of the Ds element inserted into the first exon of At1g65200, and the Ds 5' end at ~800 bp upstream of At1g66030, suggesting that ~350 kb of DNA between these two genes might have been deleted at this Ds locus (Fig. 3A). To confirm this deletion, we pollinated Col wild-type flowers with glc/GLC pollen in a Ler background and tested for the presence or absence of the Ler alleles in the F1 hybrid glc(Ler)/GLC(Col) of nine genes distributed throughout this putative deletion (see Page et al., 2004). We used the BlastDigester software (Ilic et al., 2004) to design the cleaved amplified polymorphisms (CAPS) markers for the single nucleotide polymorphisms (SNPs) in these genes between Ler and Col ecotypes that can be distinguished by restriction digestions of the PCR products. We found that for the five genes distributed in the ~215 kb of genomic DNA towards the 3' Ds, only the Col alleles were present in the F1 hybrid (Fig. 3A), indicating that this DNA is deleted in glc. The other four genes in the ~135 kb of genomic DNA towards the 5' Ds showed both Ler and Col alleles in the F1 hybrid (Fig. 3A), suggesting that this segment of DNA is either duplicated elsewhere in the Ler genome or was transposed by the Ds insertion to a new location. Further characterization was performed by Southern blot analysis of glc/GLC and wild-type genomic DNA digested with several restriction enzymes within the Ds element and the regions flanking both sides of the Ds, using DNA probes that hybridized to the Ds element or the flanking regions. The Ds-specific probe indicated only one Ds copy in glc plants (Fig. 3B). The flanking-region probes revealed the restriction patterns consistent with the predicted genomic sequences in the immediate vicinity (3-4 kb) on both sides of the Ds (see Fig. S2 in the supplementary material). These results suggest that the Ds insertion did not generate further rearrangements flanking the site of insertion.

We then investigated the possibility that the glc phenotype does not arise from the deletion but from a second site mutation linked to the identified Ds locus, possibly arising from a Ds footprint. We looked for recombinants between glc and the Ds insertion in the F1 progeny of crosses between glc/GLC male and Ler wild-type female plants. Several independent lines of two recombinant types were recovered at rates of ~1%. Type I recombinant plants were kanR indicating the presence of the Ds element (see Fig. S3A in the supplementary material), but were phenotypically wild type. Type II recombinant plants were kanS indicating the absence of the Ds

Fig. 1. Normal morphology and intact cell identities of the mature embryo sac in a SET2030 ovule. (A) Mature embryo sac with four cell types: two synergids, one egg cell and one central cell. (B-F) GUS expression of marker lines for specific cell types of glc embryo sacs in mature ovules. Central cell marker lines are FIS2:GUS (B) and MEA::GUS (C), egg cell marker lines are ET1086 (D) and ET11119 (E), and the synergid cell marker line is ET2634 (F). ccn, central cell nucleus;ecn, egg cell nucleus; scn, synergid cell nuclei. Scale bars: 50 μm.

Fig. 2. glc (SET2030) phenotypes in fertilized seeds. (A) Wild-type seed at the late globular embryo stage. (B-G) glc seeds without endosperm and with embryos arrested at various stages: one-cell (B), two-cell (C), quadrant (D), octant (E), pre-globular (F), globular (G). (H) glc 16-cell embryo with a cluster of six unequally sized nuclei/nucleoli in the endosperm. (I) High-magnification image of the globular glc embryo in G. (J) Wild-type globular embryo. Arrows indicate the single nucleus/nucleolus or nuclear/nucleolar cluster in the glc central cell. Scale bars: 50 μm.
element (see Fig. S3A in the supplementary material), but the mutant phenotype was maintained. Next, we inspected whether the deletion at the Ds locus was still present in these recombinants by examining the Ler CAPS markers spanning the deletion in F1 hybrids of the recombinants with wild-type Col, as for the original glc mutant line. We found that all Ler CAPS markers in the deletion were now present in type I recombinants but still absent in type II recombinants (see Fig. S3B in the supplementary material). These data indicated that the glc mutant phenotype is correlated with the absence of a DNA segment rather than the presence of the Ds element. We further confirmed the recovery of the deleted DNA in type I recombinant plants by Southern blot analysis with probes hybridizing to the DNA corresponding to the deletion: the band signal intensity for type I recombinant plants was in the intensity range of wild-type Ler plant and approximately twice as strong as that of the original glc mutant line and of the type II recombinant plants (see Fig. S3C in the supplementary material). Taken together, these results indicate that the glc phenotype in the original glc mutant line and in the type II recombinant lines was caused by the deletion associated with the Ds insertion, and the phenotypic rescue occurring in type I recombinants resulted from the recovery of the DNA in this deletion, possibly arising through unequal crossover (see Discussion). We also assessed the glc mutation for recombination/dominance in diploid glc/GLC gametophytes from tetraploid flowers created by colchicine treatment of the meristem of diploid glc/GLC plants. The kanR:kanS ratios of plants grown from the mixed diploid and tetraploid seeds of three independently treated plants were 2:1 (n=293), 1.5:1 (n=350) and 3.6:1 (n=327) – much higher than, and significantly different from, the 0.64:1 ratio of seeds of the original diploid glc/GLC plants (P<0.0001 in all cases), suggesting a significantly increased transmission of the glc mutant allele. This result indicates that the presence of a wild-type GLC allele in heterozygous glc/GLC gametophytes rescues the mutant effect of the glc allele. Therefore, we conclude that glc is a recessive loss-of-function mutation caused by the deletion of this DNA fragment.

Table 2. Phenotypic classes of glc, mea-1, glc mea-1, and wild-type (wt) seeds at 2.5 DAP

<table>
<thead>
<tr>
<th>Cross</th>
<th>Normal endosperm %</th>
<th>2-8 nuclei endosperm %</th>
<th>No endosperm %</th>
<th>Unfertilized ovules %</th>
<th>Collapsed ovules %</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>glc × wt</td>
<td>62</td>
<td>5</td>
<td>25</td>
<td>5</td>
<td>3</td>
<td>158</td>
</tr>
<tr>
<td>glc mea × wt</td>
<td>65*</td>
<td>8</td>
<td>23*</td>
<td>2</td>
<td>2</td>
<td>178</td>
</tr>
<tr>
<td>mea × wt</td>
<td>95</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>217</td>
</tr>
<tr>
<td>wt × wt</td>
<td>92</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>217</td>
</tr>
</tbody>
</table>

Normal-looking seeds are listed in the second column, with ‘normal endosperm’ having at least 16 endosperm nuclei and embryo stages ranging from 1-cell to pre-globular (preglob). The majority of seeds in this class were at the 8- and 16-cell stages. Mutant seeds are listed in the third and fourth columns and fall into two categories. One category includes seeds with 2-8 endosperm nuclei; the other includes seeds without any endosperm. In both categories of this mutant seed class, embryo stages range from 1- to 8-cell, with the majority at the 4- and 8-cell stage. χ² tests were performed to compare the ‘normal endosperm’ class or the ‘no endosperm’ class between glc and glc mea-1.

*χ²=0.23, P>0.6.

**χ²=0.37, P>0.5.

glc embryos are products of fertilization

The development of embryos in the absence of endosperm in glc seeds raised the question of whether glc embryos were products of fertilization, and whether double fertilization occurred in these seeds. To address the first question, we emasculated early-12-staged flowers of glc/GLC plants and examined ovules in emasculated glc/GLC pistils at 5 days after emasculation. No autonomous embryo or endosperm formed in these ovules in the absence of fertilization. Furthermore, glc embryos from glc/GLC pistils pollinated by CYCB1;1::GUS homozygous (Colon-Carmona et al., 1999; Baroux et al., 2001) pollen expressed GUS from the paternal CYCB1;1 promoter (Fig. 4A,B). This finding rules out the possibility that glc embryos develop parthenogenetically because of the fertilization signals from the arriving sperm cells without actually being fertilized. We did not observe GUS expression in endosperm of wild-type seeds nor in the single nucleus of glc seeds. As in wild-type embryos, the suspensor domain of glc embryos also properly expressed the early embryo polarity marker gene PIN7 (Friml et al., 2003) (Fig. 4C,D), indicating that glc embryos initiate normal development.

Fertilization of the glc central cell is impaired

The central cells of glc embryo sacs appeared to differentiate normally with correct cell identity, as evidenced by the GUS expression of the two central cell-specific marker lines tested, FIS2::GUS and MEA::GUS (Fig. 1B,C), in about half of the ovules from glc/GLC siliques that were hemizygous for either FIS2::GUS or MEA::GUS (119/240 and 117/250, respectively). However, glc central cells did not develop into endosperm, raising the question of whether they were fertilized. The single nucleus in the post-pollination glc central cell could be either the unfertilized homo-diploid (2n) central cell nucleus or the fertilized triploid (3n) primary endosperm nucleus. To distinguish between these two possibilities, we examined the DNA content of this nucleus in glc seeds stained with propidium iodide and
optically sectioned by confocal laser scanning microscopy (Barrell and Grossniklaus, 2005). We used the DNA contents of 15 diploid sporophytic nuclei in the integument cells of the same glc seed as the reference for the diploid DNA content, for which the 2n values range from 2C to 4C (Fig. 5, and see Table S1 in the supplementary material). We then compared the DNA content of the single nucleus in glc central cell of each seed with this diploid standard reference of the same seed.

We studied six randomly chosen glc seeds whose proembryos were at the one- or two-cell stage, two from selfed glc/GLC siliques and four from glc/GLC siliques crossed with wild-type pollen. In five glc seeds (two glc selfed seeds and three glc out-crossed seeds), the single nucleus had the DNA content of 4C (equivalent to 2n), suggesting that the central cells in these glc seeds remain unfertilized (Fig. 5A-E and see Table S1 in the supplementary material). The remaining glc out-crossed seed displayed the DNA content of 6C (equivalent to 3n) for the single nucleus (Fig. 5F, and see Table S1 in the supplementary material), indicating that this nucleus is the fertilized triploid primary endosperm. These results suggested that glc central cell can be fertilized occasionally (one out of six times in this sample).

To validate the accuracy of our DNA quantification method for nuclei, we also examined ten triploid endosperm nuclei from a fertilized wild-type seed at the one-cell embryo stage and compared their DNA contents with those of ten diploid integument nuclei of the same seed. We found that 9 out of 10 wild-type endosperm nuclei had DNA content higher than the 4C level of the integument nuclei and only one had this 4C level (Fig. 5G, and see Table S1 in the supplementary material). Of these nine nuclei, three were in the range of the 6C level (3n). None had higher than the 6C level or below 4C level, validating that our method authentically reflects the dynamic replication typical of wild-type syncytial endosperm. This result, therefore, supported the quantification of glc central cell nucleus and confirmed the non-fertilization status of the glc central cell in general, although fertilized glc central cells do occur infrequently.

### glc suppresses autonomous endosperm development of mea and msi1 in the pre-fertilization central cell

The absence of endosperm development in glc seeds is in contrast to the autonomous and over-proliferated endosperm phenotypes of the gametophytic maternal effect fis class mutants mea, fis2, fie and msi1 (reviewed by Grossniklaus, 2005). This prompted us to investigate the genetic interactions between glc and these FIS class genes in the pre-fertilization central cell. Specifically, we asked whether glc could suppress the autonomous endosperm development of fis mutants. We generated doubly heterozygous mutants of glc with
Table 3. Proportions of ovules with autonomous endosperm in single and double mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% ovules with autonomous endosperm</th>
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<tbody>
<tr>
<td>glc/ME1</td>
<td>N/A</td>
</tr>
<tr>
<td>mea-1/ME1</td>
<td>12.8 (41/319)</td>
</tr>
<tr>
<td>glc/GLC mea-1/ME1</td>
<td>6.8 (26/383)*</td>
</tr>
<tr>
<td>mea-8/MEA-8</td>
<td>28.7 (159/554)</td>
</tr>
<tr>
<td>glc/GLC mea-8/MEA-8</td>
<td>16.8 (115/684)†</td>
</tr>
<tr>
<td>msi1-3/MSI1-3</td>
<td>40.9 (208/509)</td>
</tr>
<tr>
<td>glc/GLC msi1-3/MSI1-3</td>
<td>25.5 (226/886)‡</td>
</tr>
</tbody>
</table>

Raw numbers are in parentheses. N/A, not applicable.

*χ²=7.41, P<0.01.
†χ²=25.1, P<0.001.
‡χ²=35.57, P<0.001.

mea-1 (Grossniklaus et al., 1998), mea-8 (SAIL_55_B04, ABRC stock center) or msi1-3 [previously reported as the mee70 mutant (Pagnussat et al., 2005)] and counted the number of whole-mount ovules 6-7 days after emasculation that displayed more than one nucleus in the central cell. The proportions of autonomous endosperm ovules in the single mutants of mea-1, mea-8, and msi1-3 were 12.8%, 28.7% and 40.9%, respectively (Table 3). If GLC functions were not required in the endosperm repression pathway in the absence of fertilization, these proportions should remain similar in the double mutants. Conversely, if glc suppressed autonomous endosperm of fis class mutants, the presence of glc in the double mutants should reduce the fraction of ovules with autonomous endosperm because a quarter of ovules in any silique is expected to carry both glc and mea or glc and msi1, respectively. Consistent with the second possibility, the proportions of ovules with autonomous endosperm were reduced by nearly half in the double mutants of glc with mea-1 (6.8%), mea-8 (16.8%), and msi1-3 (25.5%) (Table 3). Therefore, we conclude that GLC functions are required either downstream of the FIS class genes, or independently in addition to the FIS class genes for autonomous endosperm development in the central cell before fertilization.

glc is epistatic to mea in fertilized seeds

Although a portion of mea embryo sacs undergo autonomous endosperm development without fertilization (Table 3), mea egg cells and central cells can be fertilized. Seeds from fertilized mea-1 embryo sacs resemble wild-type seeds at early stages, although their development from the globular stage onwards is delayed and both endosperm and embryo abnormally over-proliferate and eventually abort (Grossniklaus et al., 1998). By contrast, in fertilized glc seeds, no endosperm develops and glc embryo development progresses more slowly than in the wild-type embryo, resulting eventually in embryo arrest (Table 2). Therefore, we investigated the epistasis between glc and mea in whole-mount double mutant glc mea-1 seeds fertilized by wild-type pollen at 2.5 DAP. At this time point, most seeds of mea-1 single mutant and wild-type siliques (95% and 92%, respectively) were morphologically normal, ranging from one-cell to pre-globular stages with normally proliferating endosperm (Table 2). On the other hand, the glc single mutant had 62% normal-looking seeds and 25% 'no-endosperm' seeds (Table 2). In the double mutant glc mea-1, if glc is epistatic to mea at and after fertilization, we expected the glc seed proportion to remain unchanged. Conversely, if mea is epistatic to glc, we predicted a reduction in the glc seed proportion and an increase in normal-looking seed frequency. Compared with single mutants, we found no evidence for significant changes in the double mutants, which had 65% normal-looking seeds and 23% 'no-endosperm' seeds (Table 2), indicating that glc is also epistatic to mea with respect to post-fertilization seed development. Thus, we suggest that GLC post-fertilization functions are also required either downstream of MEA or, alternatively, in an independent antagonistic pathway.

Embryonic expression of the PHE1 gene is abolished in glc seeds

PHE1, a direct downstream repression target of MEA and FIE, is expressed in both embryo and endosperm soon after fertilization, peaks at the early globular stage and declines from the late globular stage onwards (Köhler et al., 2003b). Paternal PHE1 is unaffected by MEA, but maternal PHE1 is partially repressed by maternal MEA (Köhler et al., 2003b; Köhler et al., 2005; Makarevich et al., 2006). As glc displays antagonistic effects to mea in both unfertilized ovules (Table 3) and fertilized seeds (Table 2), we explored the effect of the glc mutation on embryonic PHE1 expression in fertilized seeds by monitoring PHE1 promoter activity of either parental allele in glc seeds at the globular or early globular stage, when PHE1 is most strongly expressed (Köhler et al., 2003b; Köhler et al., 2005). To examine paternal PHE1, we pollinated glc/GLC flowers with PHE1::GUS homozygous pollen. To observe maternal PHE1, we crossed female glc/GLC PHE1::GUS/wild-type pollen. In siliques from these crosses, wild-type seeds were easily distinguished from glc seeds based on their size and the presence/absence of endosperm. In both types of cross, we found GUS expression only in wild-type but not in mutant seeds (Fig. 6), indicating that maternal GLC functions are required for the activation of both parental PHE1::GUS alleles in fertilized seeds.

Paternal embryonic expression of the RPS5a and FAC1 genes is affected in glc seeds

Although GLC is required for embryonic PHE1 expression (Fig. 6), the glc mutation does not affect embryonic CYCB1;1 and PIN7 expression (Fig. 4). As CYCB1;1 and PIN7 are expressed only in the embryo whereas PHE1 is expressed in both embryo and endosperm, we assessed the extent of the impact of GLC on two other genes, FAC1 (Xu et al., 2005) and RPS5a (Weijers et al., 2001), known to be expressed bi-parentally in both embryo and endosperm from the zygotic and two-cell embryo stage onwards, respectively. Embryos are arrested at the zygotic stage in fac1 mutant seeds when both parental alleles are disrupted (Xu et al., 2005), whereas embryo development proceeds as far as the globular and walking stick stages with normally proliferating endosperm (Table 2), indicating the embryo due to interference by intensive FAC1::GUS expression in the sporophytic integument tissue. These results suggest that maternal GLC functions are required for the expression of the paternal alleles of FAC1 and RPS5a.
We also assessed the requirement of maternal GLC functions for paternal PHE1, RPS5a, and FAC1 allele expression in the two types of recombinants described above (see Fig. S3 in the supplementary material). Similarly to the original glc mutant line, in mutant embryos of type II recombinant lines, where glc phenotype and the deletion at the Ds locus were still maintained (see Fig. S3 in the supplementary material), no paternal promoter activities of these three genes were detected (data not shown). Conversely, in embryos and endosperm of type I recombinant lines, where the phenotype was reverted to wild type and the DNA at the deletion was recovered (see Fig. S3 in the supplementary material), GUS expression reported by paternal promoters of these genes was detected in the majority of seeds as observed in seeds of wild-type plants (data not shown). Therefore, the recovery of the deleted DNA rescued not only the mutant phenotype but also the maternal activation of the paternal alleles of the examined genes, confirming that GLC functions reside within this deletion.

**DISCUSSION**

**Gametophytic maternal effects of the glc mutation**

We have identified a gametophytic maternal effect mutant glc, in which the embryo develops in the absence of endosperm, and fertilization of the central cell is impaired. The glc mutation displays an antagonistic relationship to fix class mutations in pre- and post-fertilization seed development. Furthermore, paternal expression of several genes important for embryo and endosperm development is affected by the glc mutation. As these effects manifest via the maternal glc mutant allele, GLC functions in these reproductive processes appear to be female-gametophytic, and lie within a deletion of ~215 kb at the Ds insertion site on chromosome 1. Phenotypic rescue exhibited in the recombinants where the DNA in this deletion has been recovered implies that glc is a recessive loss-of-function mutation. Our recombinant data (see Fig. S3 in the supplementary material) are consistent with an event in which the Ds insertion that generated the deletion also transposed part of the DNA (~135 kb) adjacent to the telomeric side of the deletion to a centromeric site separated by ~1 cM (see Fig. S4 in the supplementary material). This model predicts that the two types of recombinants observed will be generated at frequencies of ~1% through unequal crossover between the mutant chromosome and the wild-type chromosome (see Fig. S4 in the supplementary material).

A deletion in another mutant, *tons missing* (*tms*), which partially overlaps the centromeric side of the deletion in *glc* up to *At1g65330* (*PHE1*), results in embryo lethality (Page et al., 2004). As glc is a gametophytic mutant and primarily affects the endosperm, this overlap region containing *PHE1* cannot be the cause of glc phenotype. In addition, we were unable to complement the glc mutation using *PHE1* alone (Q.A.N. and V.S., unpublished). However, we cannot rule out the fact that the glc phenotype requires the function of more than one gene, for example, a gene such as *PHE1* that lies within the overlap with the *tms* deletion and another gene that lies outside the region of overlap.

**Communication flow between embryo and endosperm in glc early seed development**

Successful fertilized seed development requires efficient coordination and effective communication between the embryo, endosperm and seed coat. In wild-type *Arabidopsis*, the seed initiation program commences with at least three rounds of endosperm nuclear division before the zygote starts its first division (Faure et al., 2002; Mansfield and Briarty, 1990). This developmental progression could indicate that endosperm formation is a requirement for the onset of embryogenesis. As was proposed for *agl80* mutant embryo sacs, a lack of endosperm might result in an arrested zygote (Portereiko et al., 2006b). However, other lines of evidence suggest that this might not be the general rule, as the embryo can develop up to the globular or heart stage in *capulet2* (*cap2*) mutant seeds despite severely...
retarded and abnormal endosperm (Grini et al., 2002), or in wild-type seeds where four-nucleate endosperm is ablated by the expression of diphtheria toxin (Weijers et al., 2003). glc embryo development up to the globular stage in the complete absence of endosperm additionally provides an unequivocal argument for the independence of early embryogenesis from the endosperm. Therefore, at the earliest stage of seed development, communication from the early endosperm to the zygote appears not essential for the coordinated development of the embryo.

Evidence for the reverse communication – embryo-to-endosperm – has recently been reported (Nowack et al., 2006; Nowack et al., 2007). Although the zygote/embryo is not required for autonomous endosperm development as known from the fis class mutants (reviewed by Chaudhury and Berger, 2001) (Gehring et al., 2004; Grossniklaus, 2005), unfertilized endosperm proliferation in seeds having fertilized embryos suggests that, when present, the zygote/early embryo triggers a signal to the unfertilized central cell resulting in partial endosperm development (Nowack et al., 2006). Moreover, when the egg cell alone is fertilized, endosperm development of the unfertilized central cell is further promoted by the mea mutation (Nowack et al., 2006; Nowack et al., 2007). However, the apparently normally developing glc embryos fertilized by wild-type pollen (Figs 2, 4) were not accompanied by development of endosperm. The glc mutation prevents autonomous endosperm development in mea and msi1 embryo sacs, where fertilization is not a consideration (Table 3), and post-fertilization endosperm development was not significantly different in mea glc double mutant versus glc single mutant embryo sacs (Table 2). Thus, we conclude that the glc phenotype is probably due to the inability of the central cell to undergo endosperm development in glc mutant embryo sacs, rather than the failure of signaling following egg cell fertilization.

**Fertilization of the central cell is specifically impaired in the glc mutant**

Double fertilization is ubiquitous in angiosperms (reviewed by Friedman and Williams, 2004). The second fusion event between a sperm cell nucleus and a ventral canal nucleus has also been observed in the non-flowering seed plant genera *Epipedium* (Friedman, 1990; Friedman, 1992) and *Gnetum* (Carmichael and Friedman, 1995), which, together with *Welwitchia*, are collectively referred to as the Gnetales (Bowe et al., 2000; Chaw et al., 2000). To date, three angiosperm mutants of known molecular identity, *DUO1* (Rotman et al., 2005), *GCS1* (Mori et al., 2006; von Basser et al., 2006) and *NFD1* (Portereiko et al., 2006a), have been reported to disturb both fertilization events, where neither gamete fusion nor embryo and endosperm development are observed. Thus, common genetic programs for the fertilization process shared by both the egg cell and the central cell must have been compromised in these mutants.

How the fertilization event between the central cell and a sperm cell has evolved during angiosperm evolution remains unknown. It could have resulted from the co-option of existing genetic programs of the egg-sperm fertilization event or as a genetic novelty. Until now, only the cdc2a mutant has been reported to have preferential fertilization of the wild-type egg cell by the single sperm present in cdc2a mutant pollen (Nowack et al., 2006). In the glc mutant, impaired fertilization is also specific to the central cell, but here the defect arises from the female gamete. Although both wild-type sperm cells released from wild-type pollen are present in glc embryo sac and there is no evidence for sperm dimorphism in *Arabidopsis*, the egg cell is the preferred choice of fertilization. This observation suggests that there is specificity in the control of the fertilization event by each female gamete, whether it lies in the instructive signals for gamete recognition, the cell structures that facilitate plasmogamy, or the nuclear structures that enable karyogamy. Furthermore, although the glc central cell at the time the embryo sac matures appears morphologically normal with correct cell identity, its disability in accomplishing fertilization evokes the question: when is its fertilization competency established? We are currently carrying out further studies on the glc mutant to answer these questions.

**Maternal antagonism between FIS and GLC functions in endosperm and embryo development**

Sexually reproducing angiosperms repress seed development in the absence of fertilization. In *Arabidopsis*, autonomous endosperm repression is achieved by the FIS-PRC2 complex (Chaudhury et al., 1997; Grossniklaus and Vielle-Calzada, 1998; Guittion et al., 2004; Köhler et al., 2003a; Kiyosue et al., 1999; Luo et al., 1999; Ohad et al., 1996; Ohad et al., 1999; Wang et al., 2006). Maternal MEA of this complex establishes repressive histone methylation marks on the maternal allele of the target gene *PHE1*, thus repressing maternal *PHE1* expression in the central cell before fertilization (Köhler et al., 2005; Köhler and Makarevich, 2006). The suppressive effect of glc on the autonomous endosperm phenotype of fis mutants (Table 3) qualifies maternal GLC functions as a component of the positive regulation of the fertilization-independent endosperm development pathway (Fig. 8A).

The FIS genes also negatively regulate endosperm and embryo proliferation after fertilization. This is evidenced in fertilized fis seeds where the endosperm overproliferates (Luo et al., 2000; Vielle-Calzada et al., 1999; Yadegari et al., 2000; Guittion et al., 2004; Köhler et al., 2003a), *mea-1* embryos over-grow aberrantly (Grossniklaus et al., 1998) and msi1 embryos develop abnormally (Köhler et al., 2003a; Guittion and Berger, 2005; Pagnussat et al., 2005). This post-fertilization FIS function is presumably accomplished by repressing target genes that might positively regulate embryo and endosperm development, such as *PHE1* and *MEIDOS* (Köhler et al., 2003b). In wild-type fertilized seeds, maternal MEA activity partially represses the maternal *PHE1* allele, and the low level of maternal *PHE1* expression is hypothesized to result from activators not identified so far (Köhler et al., 2005; Makarevich et al., 2006). In glc seeds, this residual promoter activity of maternal *PHE1* was totally abolished (Fig. 6C,D), suggesting that GLC functions could fulfil the role of the proposed additional regulator. This requirement of maternal GLC for maternal *PHE1* activation, together with the pre- and post-fertilization epistasis between glc and mea (Tables 2, 3), designates maternal antagonism between GLC and FIS functions in seed development.

Another mutant reported to have a similar epistatic relationship with *mea* is *cap2*, which is mapped ~5 Mb telomeric of glc (Grini et al., 2002). However, the post-fertilization interactions of *PHE1* and *CAP2* are not known. The nature of the *cap2* pre-fertilization interaction with *mea* appears to be different from that of glc with *mea*. This dissimilarity is reflected in double mutant *mea cap2* seeds having the same proportion of ‘autonomous seeds’ as does *mea*, and the much more retarded *cap2* embryo development compared with glc embryos despite partial endosperm development in *cap2* seeds (Grini et al., 2002). Therefore, the different outcomes from the double mutant analysis with *mea* might arise from the different functions of GLC and CAP2.
GLC functions in the model of FIS-regulating seed development

Although maternal PHE1 is negatively regulated by maternal MEA, paternal PHE1 expression is not affected by MEA (Köhler et al., 2005). How the paternal PHE1 allele is activated in fertilized seeds has been a missing link in the downstream genetic regulatory network of the FIS genes. GLC provides a plausible candidate for this missing link because paternal PHE1 promoter activity was also eliminated in glc embryos that maternally inherit the mutant glc allele (Fig. 6A,B). The dependence of paternal RPS5a and FAC1 expression on the presence of the wild-type maternal GLC allele (Fig. 7) suggests that maternal GLC functions play a role in the activation of paternal RPS5a and FAC1 in addition to paternal PHE1. In the case of PHE1 and RPS5a (Köhler et al., 2005; Weijers et al., 2001), the observed effect of maternal glc on these paternal alleles is unlikely to be due to the developmental delay of glc embryos, since even the few persistent globular glc embryos at 4 DAP still did not show regular GUS expression from PHE1 and RPS5a paternal promoters (Fig. 6A,B, Fig. 7C-E). Nevertheless, we do not rule out the idea that the activation effect of maternal GLC functions could be an indirect influence of maternal GLC on other maternal factors that operate on the paternal alleles of these genes.

We note that the deletion in the glc mutation encompasses both PHE1 and the closely related gene PHE2 (At1g65300), as does the deletion in the tms embryo-lethal mutant (Page et al., 2004). PHE1 per se is not essential for seed development because homozygous phe1/phe1 plants have no developmental defects (Köhler et al., 2005). It cannot be concluded that lethality in tms and glc is attributable to the absence of only these two genes, because many other genes are also deleted in both mutants. Although the deletion on the maternal glc allele abolishes bi-parental PHE1 expression altogether (Fig. 6), it is unable to rescue the mea mutation because the double mutant glc mea behaves like the single mutant glc (Table 2). Yet, mea seeds where PHE1 expression level is restored to the wild-type level via antisense PHE1 under MEA promoter control or via a ddm1 demethylation background, can be partially rescued (Köhler et al., 2003b; Köhler et al., 2005). Therefore, although PHE1 alone is not necessary for seed development, it appears that in the absence of MEA activity, a certain threshold of PHE1 transcripts must not be exceeded to avoid seed abortion.

We propose that GLC functions can be integrated into the model of seed development regulated by the FIS genes as shown in Fig. 8B. Maternal GLC could operate in a pathway to promote endosperm growth independently of the repressive activity of maternal FIS genes. It is also possible that maternal GLC functions downstream of MEA in the FIS-regulated pathway for embryo development and might be partially repressed by maternal MEA, either directly or indirectly. After fertilization, embryo and endosperm development are promoted by PHE1 and other seed-growth-promoting genes, such as MEIDOS, RPS5a and FAC1. Bi-parental PHE1 and paternal RPS5a and FAC1 are activated by maternal GLC, either directly or indirectly, at least in the embryo and possibly also in the endosperm. FIS genes counteract GLC action by negatively regulating the seed growth promoting genes PHE1 and MEIDOS and possibly GLC. This counteraction prevents the unchecked and imbalanced stimulation that leads to aberrant embryo and endosperm proliferation, which ultimately results in seed abortion.

A new perspective of maternal control in early seed development: paternal allele activation by a maternal factor

Although gene expression mechanisms of paternal alleles in plant early embryogenesis have not been surveyed and documented, several mechanistic scenarios can be postulated. Paternal expression could be a carry-over from the pre-expressed state in the male gametophyte genome before fertilization. It could also be induced by the pre-programmed self-activation of the paternal alleles after fertilization. Another possibility is that paternal allele activation requires embryonic regulators derived from both parental genomes. The dependence of paternal PHE1, RPS5a and FAC1 expression on maternal GLC demonstrates that paternal allele expression of certain genes, at least in the embryo, is induced by some element(s) of the maternal genome, which is derived from the female gametophyte. Of the five genes with diverse functions that we examined for expression from the paternal allele, two exhibit detectable expression only in the embryo and not in the endosperm: CYC B1;1, which is one of the Arabidopsis mitotic cyclins (Colon-Carmona et al., 1999) and PIN7, which establishes early embryonic polarity and patterning via effecting an auxin activity gradient (Friml et al., 2003). The other three genes, PHE1 (Köhler et al., 2003b), RPS5a (Weijers et al., 2001) and FAC1 (Xu et al., 2005), also belong to different functional categories: PHE1 is a transcription factor; the remaining two are considered housekeeping genes that encode a ribosomal protein subunit (RPS5a) and an AMP deaminase (FAC1). The paternal alleles of all these three genes are expressed in both the embryo and endosperm from very early stages of seed development. Therefore, the subset of paternally expressed genes that is positively regulated by maternal factors might consist of those genes that have functions in both embryo and endosperm, and not genes that function in the embryo alone. This hypothesis is consistent with the observations that GLC functions are required maternally for endosperm but not embryo development. Further investigations of glc effects on other paternally expressed genes will be needed to define the set of genes of which paternal allele activation requires maternal GLC.

The concept of prevalent maternal control of early seed development in plants has emerged during the past years, first evidenced by the preferentially maternal expression of many genes
in seeds during the first few days after fertilization in both Arabidopsis (Vieille-Calzada et al., 2000) and maize (Grimanelli et al., 2005). The gametophytic maternal effect of the FIS genes (reviewed by Grossniklaus, 2005) and of the large class of MATERNAL EFFECT EMBRYO ARREST (MEA) genes (Pagnussat et al., 2005), of which some display this early preferentially maternal expression (Jullien et al., 2006b; Kinoshita et al., 1999; Luo et al., 2000; Vieille-Calzada et al., 1999) (Q. A. Ngo, PhD thesis, University of California, 2006), provides further support for the concept. Factors of the maternal genome can regulate other maternal factors negatively, as exemplified by the repression of maternal PHE1 by maternal MEA and FIE (Köhler et al., 2003a; Köhler et al., 2003b; Makarevich et al., 2006), or positively, as demonstrated by the activation of maternal MEA by maternal DEMETER (Choi et al., 2002; Choi et al., 2004). Moreover, the regulatory mechanism of the maternal genome crosses its own genome boundary to affect the paternal genome. Recently, an intriguing mechanism by which the maternal genome contributes to early seed development has been revealed by the negative crossregulation executed by the MEA protein produced from a maternal allele, which represses its own paternal allele via histone methylation (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006a). GLC has now added positive crossregulation to the diverse repertoire of maternal control: the product from its maternal allele activates the paternal alleles of certain other genes. In light of this positive crossregulation, the nature of zygotic and early embryonic bi-parentally expressed genes merits revisiting. The origin of maternal expression of such genes might, if examined more carefully, depend upon the maternal expression of other genes that are upstream regulatory factors. Thus, in early seed development, a maternal contribution, which originates in the female gametophyte, could ultimately control the paternal contribution.

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dosage-dependent chromatin organization. The triploid endosperm genome of Arabidopsis adopts a peculiar, parental
expression (Jullien et al., 2006b; Kinoshita et al., 1999; Luo et al.,
) providing further support for the concept. Factors
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