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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 not 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 paternal 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; Vielle-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 counteracts 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.

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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

(Institute of Planty Biology, Zürich, Switzerland), Claudia Köhler (Institute of Plant Science, ETH Zürich, Switzerland) and Jirí Friml (University of Tübingen, Germany), respectively. Except for *CYCB1;1*::*GUS* and *mea-8* lines, which were both in Columbia (Col) background and *FIS2::GUS* line in C24 background, all other lines in this study were in Landsberg *erecta* (L*er*) background. Plants with the *glc*, *msi1-3* (Pagnussat et al., 2005), *mea-1* (Grossniklaus et al., 1998) alleles were selected on 50 mg/l kanamycin, *mea-8* on 10 mg/l glufosinate and *PHE1*::*GUS* (Köhler et al., 2003b) on 20 mg/l hygromycin.

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 $MgCl_2$, 200 μM each dNTP and 10 pmol each primer. PCR parameters were as follows: 94°C for 2 minutes, 30 cycles of 94°C/30 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 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 [^{32}P]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 synergid 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).

Table 1. Transmission patterns of SET2030 (*glc*)

Cross	kanR/kanS	Expected value
glc/+ × *glc*/+	0.64 (258/405)	3
glc/+ × wt	0.15 (138/907)	1
wt × *glc*/+	0.49 (398/809)	1

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.

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 silique 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 silique. 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 pre-globular or globular stage. Embryos of mutant seeds up to the pre-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/+ flowers 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

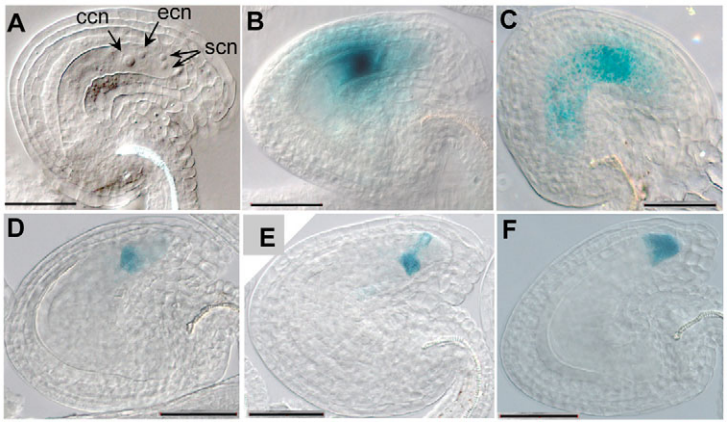

Fig. 1. Normal morphology and intact cell identities of the mature embryo sac in a SET2030 ovule. (**A**) Mature embryo sac with four cell types: two synergids, one egg cell and one central cell. (**B-F**) *GUS* expression of marker lines for specific cell types of *glc* embryo sacs in mature ovules. Central cell marker lines are *FIS2::GUS* (B) and *MEA::GUS* (C), egg cell marker lines are ET1086 (D) and ET1119 (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.

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 L*er* background and tested for the presence or absence of the L*er* alleles in the F1 hybrid *glc*(L*er*)/*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 L*er* 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 L*er* and Col alleles in the F1 hybrid (Fig. 3A), suggesting that this segment of DNA is either duplicated elsewhere in the L*er* 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 L*er* 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*

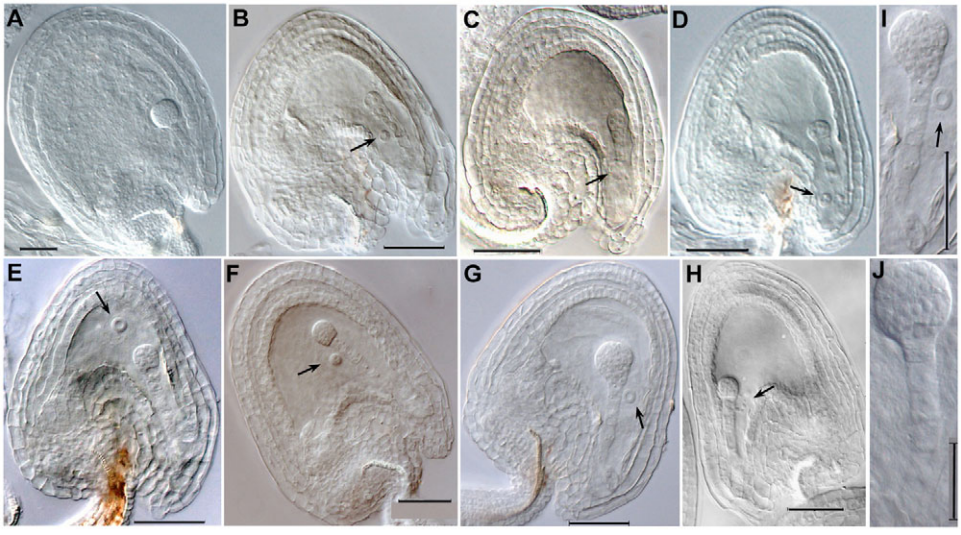

Fig. 2. *glc* (SET2030) phenotypes in fertilized seeds. (**A**) Wild-type seed at the late globular embryo stage. (**B-G**) *glc* seeds without endosperm and with embryos arrested at various stages: one-cell (B), two-cell (C), quadrant (D), octant (E), pre-globular (F), globular (G). (**H**) *glc* 16-cell 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.

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

Cross	Normal endosperm % 1 cell-preglob	2-8 nuclei endosperm % 1 cell-8 cell	No endosperm % 1 cell-8 cell	Unfertilized ovules %	Collapsed ovules %	*n*
glc × wt	62	5	25	5	3	158
glc mea × wt	65*	8	23†	2	2	178
mea × wt	95	0	0	<1	5	217
wt × wt	92	0	0	2	6	217

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. χ^2 tests were performed to compare the 'normal endosperm' class or the 'no endosperm' class between *glc* and *glc mea-1*.
*χ^2=0.23, P>0.6.
†χ^2=0.37, P>0.5.

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 L*er* 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 L*er* 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 L*er* 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 recessivity/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.

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 parthenogenically 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

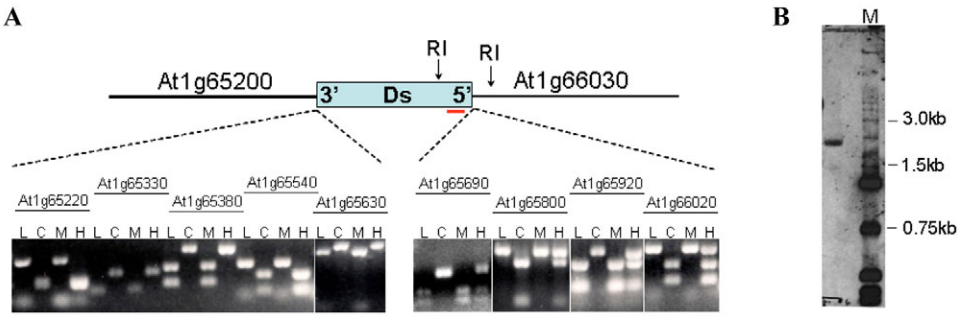

Fig. 3. *Ds* insertion locus in the *glc* mutant. (**A**) CAPS markers in the putative deletion region at the *glc* locus of the *glc*(L*er*)/*GLC*(Col) hybrid. L, wild-type L*er*; C, wild-type Col; M, *glc*(L*er*)/*GLC*(L*er*); H, F1 hybrid *glc*(L*er*)/*GLC*(Col); RI, *Eco*RI. (**B**) Southern blot of genomic DNA from *glc/GLC* plant hybridized with the probe within the *Ds* element represented by the red line in A. M, size marker.

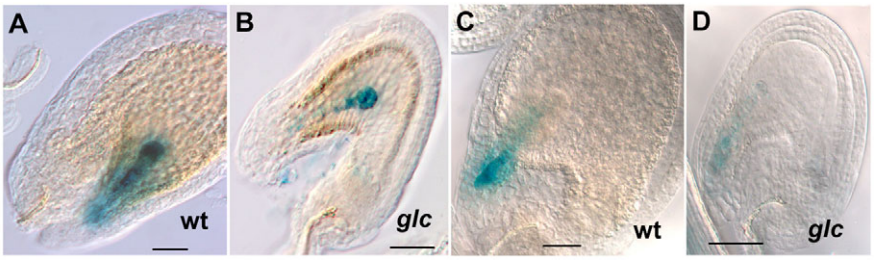

Fig. 4. Expression of paternal markers in embryos derived from *glc* egg cells. Paternal promoter activity of *CYCB1;1::GUS* (**A**,**B**) and *PIN7::PIN7-GUS* (**C**,**D**) in the pre-globular embryos 2.5 DAP of wild-type (wt) seeds (A,C) and of *glc* seeds (B,D). Scale bars: 50 μm.

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 2*n* 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 2*n*), 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 3*n*) 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 (3*n*). 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

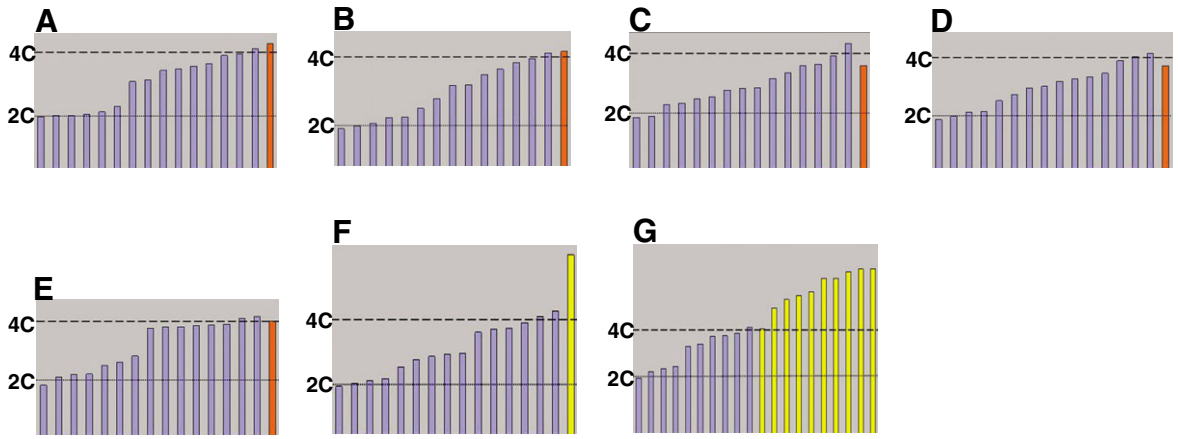

Fig. 5. DNA content of endosperm nuclei derived from *glc* central cell. DNA content of the sporophytic integument nuclei (blue bars), the single nucleus (orange or yellow bars) in *glc* seeds and the endosperm nuclei in wild-type seed (yellow bars). (**A**,**B**) *glc* selfed seeds. (**C-F**) Out-crossed *glc* seeds. (**G**) Wild-type seed. Each graph displays data from one seed. Each seed had its own diploidy reference owing to the laser-scanning settings and variation in dye penetration from seed to seed. As the integument cells of early seeds are simultaneously dividing and expanding (Haughn and Chaudhury, 2005), their nuclei have different amounts of DNA depending on where the cells are in the cell cycle. The nuclei with the lowest DNA content represent 2*n* at G1 (2*n*=2C), the nuclei with the highest DNA content represent 2*n* at G2 (2*n*=4C), and the nuclei with the DNA content between these two values represent the amount of DNA from the mother nucleus plus that from the replicating DNA strands (equivalent to 2C<2*n*<4C). For each series of 15 sporophytic nuclei of each *glc* seed, the average of the DNA contents of the three nuclei with the lowest fluorescence intensity was taken as 2C, and of the three nuclei with the highest fluorescence intensity as 4C. The *x*-axis depicts separate nuclei; the *y*-axis shows the fluorescence intensity sum of the propidium iodide-stained nuclei. Horizontal dotted lines represent the average 2C level; horizontal dashed lines represent the average 4C level.

Table 3. Proportions of ovules with autonomous endosperm in single and double mutants

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

Raw numbers are in parentheses.
N/A, not applicable.
*χ^2=7.41, $P \leq 0.01$.
†χ^2=25.1, $P \leq 0.001$.
‡χ^2=35.57, $P \leq 0.001$.

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 preglobular 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*/– flowers with 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 in *rps5a* homozygous and heterozygous mutants, respectively, the latter because of haploinsufficiency (Weijers et al., 2001). We examined the promoter activity of the *FAC1* and *RPS5a* genes in *glc /GLC* embryos, using plants carrying *FAC1::GUS* or *RPS5a::GUS* fusions. We found that in *glc/GLC* embryos, paternal expression of both *FAC1* and *RPS5a* was severely affected, whereas wild-type embryos at comparable stages showed strong expression (Fig. 7). Paternal *FAC1::GUS* was abolished (Fig. 7A,B), as was paternal *RPS5a::GUS* (Fig. 7C,D), though the latter showed sporadic expression in some *glc* embryos (Fig. 7E). On the other hand, maternal *RPS5a* promoter activity was not affected by *glc* (Fig. 7F). Expression of maternal *FAC1::GUS* could not be examined in embryos 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*.

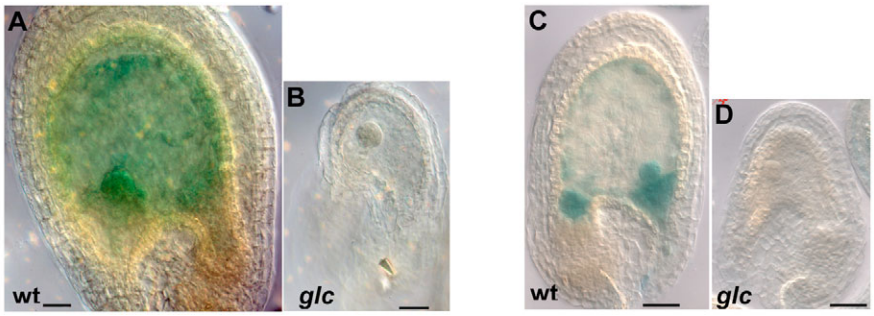

Fig. 6. *PHE1* expression in *glc* seeds. Embryonic *PHE1* expression in wild-type seeds (**A**,**C**) and *glc* seeds (**B**,**D**) 2.5-4 DAP from paternal (A,B) and maternal (C,D) *PHE1* promoter activity. (A,B) Embryos at the early globular stage. (C,D) Embryos at the pre-globular stage. wt, wild-type. Scale bars: 50 μm.

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 *fis* 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

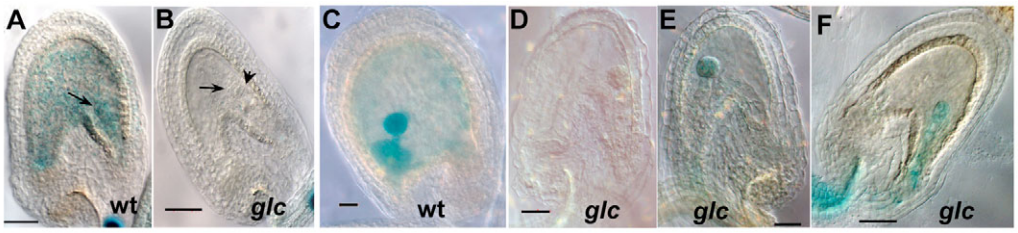

Fig. 7. Expression of paternal markers in *glc* seeds. Embryonic *FAC1* and *RPS5a* expression in wild-type seeds (**A**,**C**) and *glc* seeds (**B**,**D-F**) from the promoter activity of paternal *FAC1* (A,B), paternal *RPS5a* (C-E) and maternal *RPS5a* (F). (A,B) Embryos at the one-cell stage. (C,D,E) Embryos at the globular stage. (F) Embryo at the eight-cell stage. Arrows in A and B indicate the embryo proper. Arrowhead in B indicates the cell wall of the embryo proper. wt, wild-type. Scale bars: 50 μm.

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 *Ephedra* (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 Besser 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; Guitton 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; Guitton 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; Guitton 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.

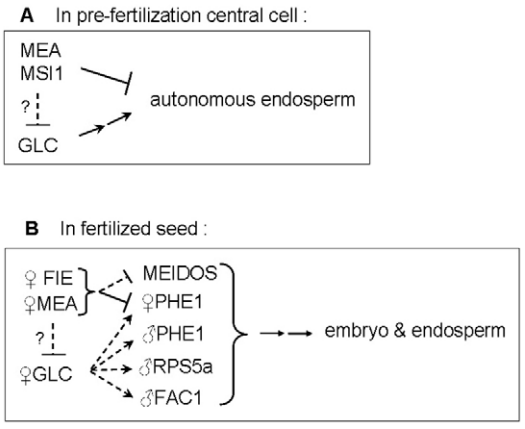

Fig. 8. Proposed *GLC* functions in the *FIS*-regulation model for endosperm and embryo development. (**A**) Before fertilization, *GLC* promotes fertilization-independent endosperm development in a separate pathway opposing *MEA* and *MSI1*. Alternatively, *MEA* and *MSI1* could prevent autonomous endosperm by repressing *GLC*. (**B**) After fertilization, maternal *GLC* directly or indirectly activates bi-parental *PHE1*, paternal *RPS5a* and paternal *FAC1* to initiate embryo and endosperm proliferation independently of *MEA-FIE* or as a downstream repression target of *MEA-FIE*. Maternal *MEA-FIE* checks and balances this cellular proliferation by repressing maternal *PHE1*, *MEIDOS* and possibly maternal *GLC*.

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* (Vielle-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* (*MEE*) 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; Vielle-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 paternal 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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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/22/4107/DC1

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