Embryo and Endosperm Development Is Disrupted in the Female Gametophytic *capulet* Mutants of Arabidopsis

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ABSTRACT

The female gametophyte of higher plants gives rise, by double fertilization, to the diploid embryo and triploid endosperm, which develop in concert to produce the mature seed. What roles gametophytic maternal factors play in this process is not clear. The female-gametophytic effects on embryo and endosperm development in the Arabidopsis *mea*, *fis*, and *fie* mutants appear to be due to gametic imprinting that can be suppressed by *METHYL TRANSFERASE1* antisense (*MET1 a/s*) transgene expression or by mutation of the *DECREASE IN DNA METHYLATION1* (*DDM1*) gene. Here we describe two novel gametophytic maternal-effect mutants, *capulet1* (*cap1*) and *capulet2* (*cap2*). In the *cap1* mutant, both embryo and endosperm development are arrested at early stages. In the *cap2* mutant, endosperm development is blocked at very early stages, whereas embryos can develop to the early heart stage. The *cap* mutant phenotypes were not rescued by wild-type pollen nor by pollen from tetraploid plants. Furthermore, removal of silencing barriers from the paternal genome by *MET1 a/s* transgene expression or by the *ddm1* mutation also failed to restore seed development in the *cap* mutants. Neither *cap1* nor *cap2* displayed autonomous seed development, in contrast to *mea*, *fis*, and *fie* mutants. In addition, *cap2* was epistatic to *fis1* in both autonomous endosperm and sexual development. Finally, both *cap1* and *cap2* mutant endosperms, like wild-type endosperms, expressed the paternally inactive endosperm-specific *FIS2* promoter *GUS* fusion transgene only when the transgene was introduced via the embryo sac, indicating that imprinting was not affected. Our results suggest that the *CAP* genes represent novel maternal functions supplied by the female gametophyte that are required for embryo and endosperm development.

MATERNAL effects are fairly common in genetically tractable animals, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, and maternal-effect mutations have allowed the identification of genes whose products play important roles in setting the stage for embryo development (Johnston and Nüsslein-Volhard 1992). By contrast, the evidence for maternal effects in higher plants is rather scant, due to the plant-specific alternation of generations, with a haploid gametophyte generation occurring between two successive diploid sporophytic generations. In Arabidopsis, one of the female meiotic products, the megaspore, undergoes three rounds of nuclear divisions followed by cellularization, which results in a seven-celled female gametophyte, or embryo sac (Mansfield *et al.* 1991; Webb and Gunning 1994; Schneitz *et al.* 1995; Christensen *et al.* 1997). At the micropylar end where the pollen tube delivers the two sperm cells, the egg cell is flanked by two synergids that assist in fertilization. A large central cell that is diploid due to the fusion of two haploid nuclei separates the egg cell from the three antipodal cells of unknown function that occupy the chalazal end and degenerate before fertilization. Double fertilization of egg cell and central cell initiates development of the diploid embryo and the triploid endosperm, respectively. These two organisms develop in parallel in a coordinated interplay between sporophytic and gametophytic tissues to produce the mature seed.

Embryo and endosperm development are fundamentally different. In Arabidopsis, early embryo development is characterized by an invariant pattern of cell divisions and differentiation (Goldberg *et al.* 1994; Laux and Jürgens 1997; Harada 1999; Jürgens 2001). The endosperm initially undergoes synchronous nuclear divisions, but by the time of cellularization, different domains have been established by morphological criteria, mitotic activity, and reporter gene expression patterns (Webb and Gunning 1991; Berger 1999; Brown *et al.* 1999; Boisnard-Lorig *et al.* 2001). In Arabidopsis and most other angiosperms, the endosperm is largely consumed during embryogenesis, suggesting a nutritive function (Lopes and Larkins 1993). However, the endosperm has also been suggested to have roles in the regulation of embryo size and fruit development and may function as a check point for the initiation of sexual reproduction (Lopes and Larkins 1993; Hong *et al.* 1996; Ohad *et al.* 1996).

To what extent embryo and endosperm development

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require maternal cues is a matter of speculation since both somatic embryogenesis and endosperm development can occur *in vitro* in the absence of maternal tissue (Zimmerman 1993; Kranz *et al.* 1998). However, there is genetic evidence for both sporophytic and gametophytic maternal effects. The Arabidopsis *SHORT INTEGUMENT1* (*SIN1*) gene is required maternally in the ovule for proper embryo development, regardless of embryo or endosperm genotype (A. Ray *et al.* 1996; S. Ray *et al.* 1996). In barley, the maternal-effect mutations *shrunken endosperm* affect the endosperm in a similar manner (Felker *et al.* 1985). Sporophytic maternal effects on the endosperm have also been shown by ovule-specific downregulation of the petunia MADS box genes *FLORAL BINDING PROTEIN 7* (*FBP7*) and *FBP11* (Colombo *et al.* 1997).

The inaccessibility of the female gametophyte has been a hindrance for large-scale genetic screens and, until recently, only a few female gametophytic mutations have been described (Redei 1965; Kermicle 1971; Patterson 1994; Vizir *et al.* 1994; Vollbrecht and Hake 1995). However, more recent screens have resulted in a growing mutant collection that yields new insights into female-gametophyte development and function (Feldmann *et al.* 1997; Christensen *et al.* 1998; Howden *et al.* 1998; Grini *et al.* 1999; Vielle-Calzada *et al.* 2000; for reviews, see Drews *et al.* 1998; Grossniklaus and Schneitz 1998). Some genes have been shown to be expressed in the female gametophyte before fertilization (Springer *et al.* 1995; Kranz and Dresselhaus 1996; Nadeau *et al.* 1996; Perry *et al.* 1996; Vielle-Calzada *et al.* 2000; Cordts *et al.* 2001). The *PROLIFERA* (*PRL)* gene encodes an *MCM* family protein that regulates replication during G_1 phase of the cell cycle. β-Glucuronidase (GUS) activity from a *PRL::GUS* transgene accumulates in the central cell nucleus before fertilization, and a fraction of mutant *prl* embryo sacs arrest at G_1 checkpoints during syncytial endosperm development (Springer *et al.* 2000).

Female-gametophytic maternal effects on embryo and endosperm development have been demonstrated by mutations in the Arabidopsis *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT SEED* (*FIS*), and *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) genes (Ohad *et al.* 1996; Chaudhury *et al.* 1997; Grossniklaus *et al.* 1998). The *mea*, *fis*, and *fie* mutants show autonomous seed development, suggesting that the affected genes normally repress embryo and endosperm development in the unfertilized ovule. In addition, these genes are subject to imprinting such that only their maternal alleles are expressed whereas their paternal alleles are inactive during early seed development. The developmental consequence of imprinting is suppressed by activating additional factors in the paternal genome (Kinoshita *et al.* 1999; Kiyosue *et al.* 1999; Luo *et al.* 1999, 2000; Vielle-Calzada *et al.* 1999; Vinkenoog *et al.* 2000; Yadegari *et al.* 2000). Although the MEA, FIS, and FIE gene products are present in the egg and central cell before fertilization, the time of their requirement for embryo and endosperm development is not known.

Two separate approaches have been employed to isolate mutants affected in gametophyte development or function. Direct screens are based on seed-abortion phenotypes or transgene reporter gene expression in the female gametophyte (Springer *et al.* 1995; Christensen *et al.* 1998; Vielle-Calzada *et al.* 2000; Sorensen *et al.* 2001). By contrast, linkage-based screens detect gametophytic mutations by the altered segregation of linked morphological markers or transgene-borne resistance markers (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998; Grini *et al.* 1999). The linkage-based strategy enables direct mapping of identified mutants as well as the ability to distinguish between gametophytic and dominant maternal sporophytic effects.

To analyze the role of the female gametophyte in fertilization, we performed a linkage-based screen for mutants that were functionally impaired but did not display an easily scorable embryo sac phenotype. Our procedure was based on the assumption that recessive marker mutations closely linked *in trans* to a newly induced female gametophytic mutation would give up to 50% marker progeny rather than the Mendelian 25%. We used the multiply marked *mm1* line that carries five visible mutations at ~20-cM intervals on chromosome 1 (Grini *et al.* 1999). M2 families of mutagenized heterozygous *mm1* seeds were examined for increased frequencies of *mm1* markers. Among the lines segregating >40% marker frequencies we identified two mutants that had gametophytic maternal effects on embryo and endosperm development.

MATERIALS AND METHODS

Plant strains and growth conditions: *Arabidopsis thaliana* (L.) Heynh. var. Landsberg *erecta* was used as wild type (WT) unless indicated otherwise. The *mm1* marker line is homozygous for *angustifolia* (*an*), *distorted1* (*dis1*), *eceriferum5* (*cer5*), *apetala1* (*ap1*), and *glabra2* (*gl2*; Grini *et al.* 1999). The tetraploid *U408* line was in the Landsberg *erecta* background (M. Hülskamp, unpublished results). The *FIS2::GUS* and the *MET1 a/s* transgene constructs were in the C24 ecotype background. The *ddm1-2* ecotype was *Columbia* and it had been backcrossed to *Columbia* for eight successive generations. Progeny of selfed *ddm1-2/DDM1-2* plants were genotyped by an allele-specific PCR test using dCAPS primers *DDM1f* (5′-GAGATCTCTA CCCTCCTGT-3′) and *ddm1-2dRsa* (5′-TGAGCTACG–AGCCA TGGGTTTGTGAAACGTA-3′), as described by Yadegari *et al.* (2000). Digestion of the PCR fragments with *Rsa*I restriction endonuclease and separation on a 4% agarose gel yields an ~130-bp band for the *ddm1-2* allele.

All seeds were germinated on a mixture of soil and sand after 4 days of vernalization at 4° and grown under long-day conditions (18 hr light/6 hr dark) at 20°. *fis1* homozygous seed and progeny of crosses involving maternal *fis1* were germinated on MS medium (Murashige and Skoog 1962). *FIS2::GUS* transgene plants were selected on MS medium containing

50 μg/ml kanamycin. Seedlings were transferred to soil after 1 week and kept at high humidity for one additional week.

Mutagenesis screen and genetic characterization of mutants: Heterozygous seed from the *mm1* marker line were mutagenized with ethyl methanesulfonate as described previously (Mayer *et al.* 1991; Grini *et al.* 1999). M2 plants were screened for distorted segregation of the *mm1* morphological markers (for details, see Grini *et al.* 1999). Lines segregating >40% of two adjacent *mm1* markers were rescreened and checked for aborted seed development. For the genetic analysis of mutants, split *mm1* marker lines were used. These lines were homozygous for *an dis1* or for *ap1 gl2*. *cap1* and *cap2* were backcrossed to *Ler* three times and reintroduced to the split *mm1* marker lines. For the genetic analyses, reciprocal cross data from independent lines were pooled only when both outcrosses and selfing were done. Plants used for crosses or phenotypic analysis were rescreened for increased marker frequencies.

Histology: For whole-mount preparations of fertilized or autonomous ovules, siliques were dissected with hypodermic needles and carpel walls were removed so that all ovules remained connected to the placenta. This dissection technique enabled the position of each ovule in the silique to be scored. Dissected siliques were fixed on ice in FAA [10:7:2:1 EtOH:distilled water:acetic acid:formaldehyde(37%)] for 30 min, hydrated in a graded EtOH series to 50 mm $NaPO_4$ buffer, pH 7.2, and mounted on microscope slides in a clearing solution of 8:2:1 chloral hydrate:water:glycerol (ClH). The specimens were allowed to clear for 1 hr at 4° before inspection. Embryo sac phenotypes were inspected in methyl benzoate-cleared whole-mount ovule preparations. Ovules were stained with Mayer's Hemalaun and processed as described by Schneitz *et al.* (1995). Scanning electron microscopy studies were performed as described previously (Hülskamp *et al.* 1995a). Aniline blue visualization of pollen tubes was performed as described (Hülskamp *et al.* 1995b). The GUS assay was performed after a modified protocol from Schoof *et al.* (2000). Plant material was prefixed in ice-cold 90% acetone for 10 min, rinsed for 10 min in staining buffer (50 mm $NaPO_4$, pH 7.2; 2 mm potassium-ferrocyanide; 2 mm potassium-ferricyanide; 0.1% Triton X-100; 2 mm X-Gluc) with no substrate and incubated in staining buffer at 37° for 3–5 hr. Following a graded EtOH dehydration series to 50% EtOH, the material was postfixed in FAA on ice for 30 min and hydrated in an EtOH series to 50 mm $NaPO_4$ buffer and mounted on microscope slides in ClH. Confocal scanning laser microscopy (CLSM) and Feulgen staining were performed as described by Braselton *et al.* (1996).

Microscopy and processing of images: Light microscope preparations were examined using a Zeiss Axiophot microscope with differential interference contrast optics and epifluorescence. Photographs were taken on Kodak Ektachrome 64T or PROVIA 400 color films or with a Nikon Coolpix 990 digital camera. Scanning electron microscopy was performed with a HITACHI S 800 microscope. CSLM was performed with a Leica microscope equipped with UV light and Kr/Ar laser. Microscopic images were processed using Adobe Photoshop 6.0 and Adobe Illustrator 9.0 software.

Mapping with flanking markers: Genetic distances between flanking markers and *cap1* or *cap2* were calculated using the formula $p = 1 - 2M$, where M is the frequency of the flanking markers. The recombination frequencies were normalized relative to the size of the interval (Grini *et al.* 1999). To determine the penetrance of the mutant alleles, *cap1/CAP1* and *cap2/CAP2* were crossed with wild-type pollen donors and their F_1 progeny were checked for the *cap* ovule phenotype (see Figure 1A). More than 100 F_1 plants from each cross were inspected and none showed the *cap* phenotype, suggesting that penetrance was nearly complete.

Molecular mapping: *cap1* and *cap2* mapping populations were made by outcrosses with the Niederzenz (Nd-0) and Columbia (Col-0) ecotypes, respectively. Heterozygous mutants were crossed as male partners to wild-type plants. F_1 plants phenotyped for *cap1* or *cap2* were paternally re-outcrossed with *Nd-0* or *Col-0*. The resulting F_2 mapping populations were grown on soil and phenotypes were determined for all plants. In this crossing scheme, the mapping population consisted only of male meiotic events and the maternal lethality of the mutations did not affect the population. Genetic distance (p) was the same as recombination frequency (Rf, $p = Rf$). DNA was isolated by a modified cetyltrimethylammonium bromide miniprep protocol as described by Stewart and Via (1993). Basic molecular biology techniques were performed according to Sambrook and Russell (2001).

Mapping was performed with cleaved amplified polymorphic sequences (CAPS) or simple sequence length polymorphisms as described previously (Hauge *et al.* 1993; Konieczny and Ausubel 1993; Bell and Ecker 1994) or by the *Arabidopsis* Information Resource (http://www.Arabidopsis.org). *cap1* was mapped in a population of 108 chromosomes. On the telomere side, 7 recombinants were found for marker nga59 [on bacterial artificial chromosome (BAC) T25K19], 3 recombinants for marker T2,5 (on BAC T7I23; Folkers *et al.* 2002), 1 recombinant for marker O846a (on BAC F19P19), and no recombinants for marker m488 (on BAC T25N20; Lukowitz *et al.* 1996). On the centromere side, 17 recombinants were found for marker m59 (on BAC F20D23), 5 recombinants for marker G5957 (on BAC T27G7; Lukowitz *et al.* 1996) and no recombinants for m488. The *O846a–G5957* interval spans a region of ~1.5 Mb corresponding to ~5–7 cM. The genetic distance between *cap1* and the PCR markers was ~6 cM [(5 + 1)/108]. The *cap2* population consisted of 102 meiotic events. On the centromere side, 14 recombinants were found for marker nF5I14 (on BAC F5I14), 5 recombinants for marker nga111 (on BAC F28P22), 2 recombinants for marker ADH (on BAC T14N5), and no recombinants for dSNP142 (on BAC F18B13), a CAPS marker we made from the single nucleotide polymorphism (SNP) 142 (SNP142). This marker detected 2 independent recombinants on the telomere side. dSNP142 primers were dSNP142F 5′-CGGGGACATCTTGACGGCTT-3′ and dSNP142R 5′-TGCTCCGATACTGAACTCGTGGC-3′. Digestion of the 933-bp PCR fragment with *Ssp*I restriction endonuclease and separation on a 2% agarose gel yields 524- and 409-bp bands for the Col-0 ecotype. In the L*er* ecotype, the 409-bp band is cut into 238 and 171 bp. The ADH-SNP142 interval spanned ~1.2 Mb corresponding to ~3–5 cM, in good accordance with the genetic distance found between the PCR markers and *cap2* ($2 \times 2/102 = 4$ cM). The flanking marker *GL2* is located on BAC F19K16, adjacent to F18B13, which supports the genetic mapping data for *cap2* within the *ap-gl2* interval.

RESULTS

Isolation and genetic characterization of the two novel female gametophytic mutants *capulet1* and *capulet2*: In a linkage-based screen for increased transmission of recessive morphological markers on chromosome 1, we isolated two novel mutants termed *capulet* after Shakespeare's *Romeo and Juliet* (see materials and methods for screen details). As shown in Table 1, selfing of *capulet1* (*cap1*) and *capulet2* (*cap2*) gave increased frequencies of the flanking marker pairs *an dis1* and *ap1 gl2*, respectively (Table 1), suggesting that their wild-type

TABLE 1

Segregation of flanking markers upon self-pollination and in reciprocal crosses with the *mm1* marker line

	Flanking marker[a]					
Line	*an* (%)	*dis1* (%)	*cer5* (%)	*ap1* (%)	*gl2* (%)	*N*
cap1/CAP1 selfed	42.0	36.9				3492
cap1/CAP1 × *mm1*[b]	88.0	83.5				417
mm1 × *cap1/CAP1*[b]	49.3	46.1				371
cap2/CAP2 selfed				42.5	49.3	1722
cap2/CAP2 × *mm1*[c]				76.0	83.9	634
mm1 × *cap2/CAP2*[c]				54.6	57.9	663

Segregation data from self- and reciprocal crosses with the *mm1* marker line. The female parent is listed first in all crosses presented.

[a] A split *mm1* line was used for the reciprocal crosses (see MATERIALS AND METHODS).

[b] *mm1* markers for *CAP1* outcross were *an dis1*.

[c] *mm1* markers for *CAP2* outcross were *ap1 gl2*.

alleles linked to the *cap* mutations were not transmitted through the female or through the male gametophyte. Reciprocal backcrosses of *cap/CAP* heterozygous plants with *mm1* marker plants revealed that the *cap1* and *cap2* mutant alleles were specifically blocked in their transmission through the female gametophyte (Table 1). The map positions of *cap1* and *cap2* were calculated from flanking marker segregation data (see MATERIALS AND METHODS). *cap1* mapped within the *an-dis1* interval ~8 cM from *an* and 13 cM from *dis1* whereas *cap2* mapped near the bottom end of chromosome 1, ~18 cM south of *ap1* and 2 cM north of *gl2*. These results were confirmed by mapping against molecular markers. *cap1* was placed between PCR marker *O846a* on BAC *F19P19* and marker *G5957* on BAC *T27G7* (see MATERIALS AND METHODS for details) and was thus separated from the closely linked maternal-effect mutant *medea* by the marker *O846a* (GROSSNIKLAUS *et al.* 1998). *cap2* was mapped within an interval defined by the PCR marker *ADH* on BAC *T14N5* and a CAPS marker made from *SNP142* on BAC *F18B13* (see MATERIALS AND METHODS for details). In summary, the female-gametophyte defects of *cap1* and *cap2* appear to result from single-locus genetic lesions.

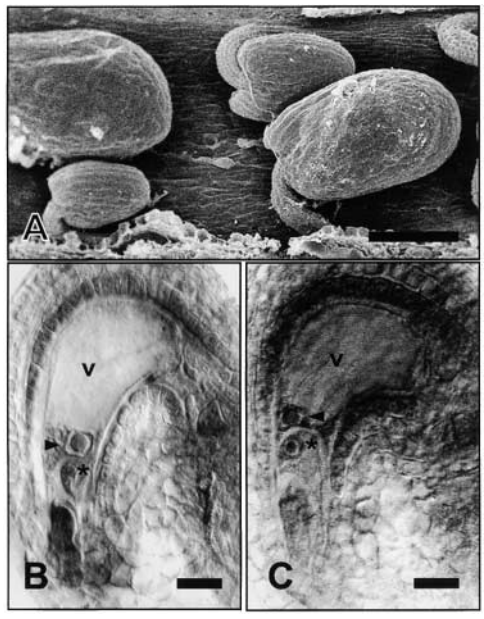

FIGURE 1.—Embryo sac and seed development in *cap* mutants. (A) Two classes of developing ovules in a *cap1/CAP1* silique at 3 DAP as shown by scanning electron micrograph. Bar, 100 μm. (B and C) Mature embryo sacs from *CAP2* control (B) and *cap2/CAP2* (C) siliques. Optical sections were obtained from whole-mount preparations of ovules. Asterisk, egg cell nucleus; arrowhead, central cell nucleus; v, vacuole. Bars, 20 μm.

***cap* mutant embryo sacs are morphologically normal but do not support embryo and endosperm development:** *cap/CAP* heterozygous plants displayed a reduced seed set of ~50% (Figure 1A). To examine whether the *CAP* genes are required for the development of the female gametophyte, we inspected whole-mount preparations of ovules from unpollinated mature siliques. In Arabidopsis, the mature embryo sac consists of an egg cell, a central cell, two synergid cells, and three degenerated antipodal cells (WEBB and GUNNING 1990, 1994; MANSFIELD *et al.* 1991; SCHNEITZ *et al.* 1995; CHRISTENSEN *et al.* 1997). By morphological criteria, embryo sac development in *cap/CAP* ovules was indistinguishable from wild type (Figure 1, B and C), and in both, <1% of embryo sacs were degenerated (n = 345 and 251, respectively). These results suggest that *CAP1* and *CAP2* genes are required for female-gametophyte function in pollen tube guidance, fertilization, or postfertilization processes.

A functional ovule and embryo sac are required for correct pollen tube guidance (HÜLSKAMP *et al.* 1995b; RAY *et al.* 1997; SHIMIZU and OKADA 2000). We examined how many ovules attracted pollen tubes in heterozygous *cap1/CAP1* and *cap2/CAP2* plants that had been

TABLE 2

Seed development in *capulet* and in reciprocal crosses with wild type

Line	WT development: Developed embryo (%)	WT development: Undeveloped embryo sac (%)	Aborted embryo development (%)	*N*
Ler × *Ler*	92.8	6.7	0.5	1545
cap1/+ × *cap1*/+	54.0	7.8	38.2	589
cap2/+ × *cap2*/+	49.9	7.0	43.1	685
cap1/+ × *Ler*	48.3	9.2	42.5	513
cap2/+ × *Ler*	46.9	9.9	43.2	574
Ler × *cap1*/+	92.2	7.6	0.2	459
Ler × *cap2*/+	92.1	6.9	1.1	277
cap1/+ unpollinated	0.0	100.0	0.0	734
cap2/+ unpollinated	0.0	100.0	0.0	547

Embryo and endosperm phenotypes were determined by whole-mount clearing preparations of ovules. Embryo and endosperm stages were determined for each ovule. The female parent is listed first in all crosses presented. See RESULTS and MATERIALS AND METHODS for further details.

pollinated with wild-type pollen. Ovules were inspected 20–24 hr after pollination (HAP), using an aniline-blue squash technique (see MATERIALS AND METHODS). Pollen tubes were observed at similar frequencies as wild-type controls (88% for both mutants, $N = 495$ and 153, respectively, as compared to 90% in wild type, $N = 240$). Thus, the *CAP* genes are not required for female-gametophyte function before fertilization.

Whole-mount preparations of ovules 12–60 HAP revealed that >90% of the embryo sacs had initiated embryo and endosperm development in the *cap* mutants (Table 2). However, embryo and endosperm were subsequently aborted in ~50% of the seeds (Table 2). Reciprocal testcrosses between *cap1/CAP1* or *cap2/CAP2* plants and wild-type plants revealed that abortion of embryo and endosperm at 1–5 days after pollination (DAP) occurred only when the maternal plant was *cap/CAP* heterozygous (Table 2) and the lethal phenotype was the same as from selfed *cap/CAP* plants (see below). Taken together, these data strongly suggest that the *cap* mutants are female-gametophyte mutants displaying maternal effects on embryo and endosperm development.

Early embryo and endosperm development of *cap* mutant embryo sacs: Following double fertilization of wild-type embryo sacs, endosperm development is initiated in the central cell before the first embryo division. The endosperm undergoes three rounds of synchronized syncytial nuclear divisions (Figure 2A). Some nuclei migrate toward the chalazal pole where they form a common cytoplasmic pocket termed chalazal cyst or chalazal endosperm (CZE; MANSFIELD and BRIARTY 1990a; BERGER 1999; BROWN *et al.* 1999; BOISNARD-LORIG *et al.* 2001). Initial endosperm development is accompanied by an elongation of the zygote and the migration of the zygote nucleus toward the apex. Concurrent with the fourth syncytial endosperm mitosis, the zygote divides asymmetrically to produce a suspensor precursor cell and the one-cell embryo proper. Following two successive longitudinal divisions (Figure 2, C and E), the four-cell (quadrant) embryo divides transversely to form the eight-cell octant embryo (Figure 3G; MANSFIELD and BRIARTY 1991; JÜRGENS 2001). At this stage, the endosperm has gone through seven rounds of free nuclear division and is composed of ~100 nuclei (Figure 3G). The syncytial mitoses are no longer synchronous although nuclear divisions are coordinated locally within the central peripheral endosperm (PEN), the micropylar peripheral endosperm (MCE), and the CZE. One or two consecutive syncytial divisions commence in the MCE and PEN and as the embryo reaches the heart stage (Figure 3H) cellularization takes place in the endosperm, initiated from the MCE surrounding the embryo. The CZE remains syncytial, containing nuclei of different sizes (MANSFIELD and BRIARTY 1990b; BROWN *et al.* 1999; BOISNARD-LORIG *et al.* 2001).

To determine the maternal-effect phenotypes of *cap1* embryo and endosperm, we analyzed whole-mount preparations of ovules from *cap1/CAP1* siliques pollinated with wild-type pollen during development from the zygote to the early heart stage. Although phenotypes were variable, developmental arrest of embryo and endosperm was generally restricted to a relatively small period of development. *cap1* embryo sacs were already abnormal at the zygote stage, which corresponds to the second or third syncytial endosperm mitosis in wild type (Figure 2A). The zygotes appeared less elongated and the endosperm contained a single enlarged nucleus (Figure 2B). Approximately 29% of mutant embryos were arrested as zygotes ($N = 257$, Table 3). Most mutant embryo sacs contained 1–2 or 4–8 endosperm nuclei (Table 3). About 41% of mutant zygotes were able to divide asymmetrically to produce one-cell proembryos (Table 3) although this division was delayed compared to wild-type embryos (Figure 2, C and E; Figure 3H). When

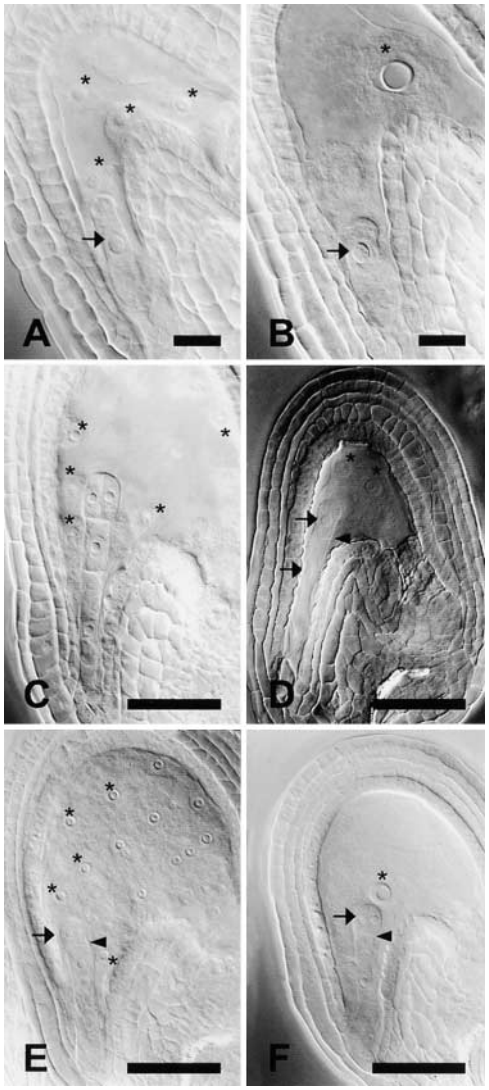

FIGURE 2.—*cap1* embryo and endosperm phenotypes. Comparisons of wild-type (A, C, and E) and *cap1* mutant ovules (B, D, and F) were made at the same time after fertilization; staging refers to wild type only. (A and B) Zygote stage. In B, only one enlarged endosperm nucleus is in arrested *cap1* mutant (asterisk). (C and D) Two-cell stage with a few enlarged endosperm nuclei in *cap1* arrested at elongated-zygote stage. (E and F) Four-cell stage with no cell wall formed between nuclei in apical cell of arrested *cap1* mutant; wild-type embryo (E) is not in focal plane. Asterisk, endosperm nucleus; arrow, embryonic nucleus; arrowhead, cell wall from division of zygote. Optical sections were obtained from whole-mount preparations of ovules from *cap1/CAP1* siliques crossed with wild-type pollen. Bars: A and B, 20 μm; C and D, 40 μm; E and F, 50 μm.

arrested at the one-cell stage, mutant embryos were most frequently associated with endosperms containing 4–8 nuclei. Approximately 23% of mutant embryo sacs lacked endosperm nuclei, which were probably degraded (Table 3). In 13% of *cap1* embryo sacs, one-cell proembryos underwent nuclear division but no cell wall was formed between the daughter nuclei (Table 3; compare Figure 2, C and F). No mutant embryos were detected beyond this stage. In general, there was a close correlation between developmental progress of the embryo and endosperm (Table 3). Most two-nucleate *cap1* embryos were surrounded by endosperm with 4–24 nuclei (Table 3) although endosperm development was often delayed as compared to their embryo partners (Figure 2F). Moreover, within an embryo sac, endosperm nuclei were always of the same size, with no typical chalazal cyst formed in endosperms with three or more syncytial mitoses. Thus, by morphological criteria, both embryo and endosperm development were affected in *cap1* embryo sacs.

In comparison to *cap1*, *cap2* embryo sacs generally did not support endosperm development beyond two syncytial mitoses whereas embryo development was delayed as compared to *CAP2* controls or arrested at various developmental stages. When nearly 80% of the *CAP2* embryos were at the four- or eight-cell stage, ~66% of the *cap2* embryos had not reached the four-cell stage (Table 4A; compare Figures 2E and 3G with Figure 3, A and B). As development progressed, older stages of *cap2* embryos were observed (Table 4B; Figure 3, C–F). Although *cap2* embryos were generally larger than *CAP2* embryos of the same age, their patterns of cell divisions were fairly normal. However, as wild-type embryos reached the heart stage, the proportion of *cap2* embryo sacs with degenerated embryos increased dramatically (Table 4B).

The development of *cap2* mutant endosperm was abnormal from the zygote stage onward (Table 4C). The number of endosperm nuclei ranged from 1 to 12, indicating that only very few rounds of nuclear division occurred (compare Figure 3, A–F, with Figure 3G). As *CAP2* development progressed, the proportion of degenerated *cap2* endosperms increased to ~80% (Table 4C). *cap2* endosperms had nuclei of different sizes and shapes, in contrast to the homogenous population of nuclei in *CAP2* and also in *cap1* endosperms (Figure 3, B–D and J; compare with Figure 3I). A chalazal cyst was not observed in *cap2* endosperms. A rare feature of *cap2* endosperms was the occurrence of domains with multiple nuclei of different sizes and shapes ("multiple" in Table 4C; compare Figure 3I with Figure 3J). These assemblies were surrounded or encapsulated by "walls" or membrane-like structures. No cellularization of *cap2* late stage endosperms was observed. However, premature cellularization of endosperms with 8 or fewer nuclei occurred at a low frequency (Figure 3, K and L). In these rare cases, all endosperm nuclei were in a different focal plane from that of the embryo, and interestingly no "cell compartments" were binuclear (Figure 3, K and L). In conclusion, *CAP2* appears to be required in the endosperm from the first syncytial mitosis. The variable enlargement of nuclei as well as the premature cellularization phenotype may suggest interference with cell-cycle regulation in the syncytial endosperm (see DISCUSSION).

The *cap* mutant phenotype does not depend on *CAP* gene dosage in the endosperm and embryo: To deter-

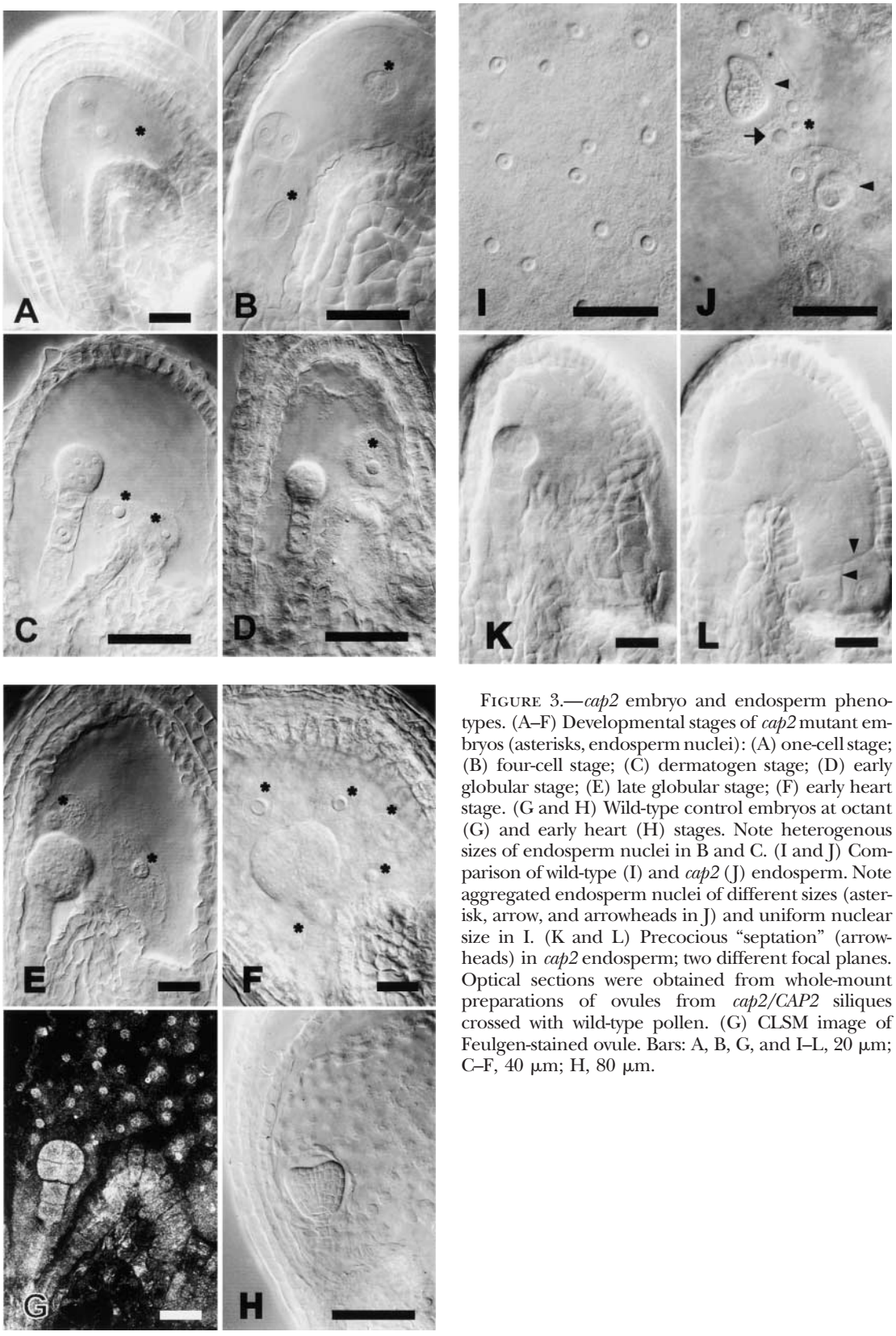

FIGURE 3.—*cap2* embryo and endosperm phenotypes. (A–F) Developmental stages of *cap2* mutant embryos (asterisks, endosperm nuclei): (A) one-cell stage; (B) four-cell stage; (C) dermatogen stage; (D) early globular stage; (E) late globular stage; (F) early heart stage. (G and H) Wild-type control embryos at octant (G) and early heart (H) stages. Note heterogenous sizes of endosperm nuclei in B and C. (I and J) Comparison of wild-type (I) and *cap2* (J) endosperm. Note aggregated endosperm nuclei of different sizes (asterisk, arrow, and arrowheads in J) and uniform nuclear size in I. (K and L) Precocious "septation" (arrowheads) in *cap2* endosperm; two different focal planes. Optical sections were obtained from whole-mount preparations of ovules from *cap2/CAP2* siliques crossed with wild-type pollen. (G) CLSM image of Feulgen-stained ovule. Bars: A, B, G, and I–L, 20 μm; C–F, 40 μm; H, 80 μm.

mine whether the number of *CAP* gene copies, rather than the parental origin of the *cap* allele, may be critical for embryo and endosperm development in *cap* mutants, we crossed *cap/CAP* diploid ($2n$) plants with tetraploid ($4n$) wild-type pollen donors. Fertilization of *cap* embryo sacs with *CAP/CAP* pollen gave *cap/cap/CAP/CAP* tetraploid endosperms and *cap/CAP/CAP* triploid embryos. Similar wild-type crosses have been reported

TABLE 3

Embryo and endosperm developmental arrest in *cap1* embryo sacs

Stage of embryo arrest[a]	Frequency[b] (%)	Endosperm nuclei[a,c] (%) Not visible[d]	1–2	4–8	12–16	>16	*N*
Zygote	29	12	36	48	1	1	74
One-nucleate proembryo	41	23	14	53	6	1	106
Two-nucleate proembryo	13	40	8	16	18	8	34
Developed ovule, collapsed	17	100	—	—	—	—	43
Mean frequency		29	18	39	6	2	257

Ovules from *cap1/CAP1* females outcrossed with wild type were analyzed in clearing preparations.

[a] Wild-type embryos from the same cross were in eight-cell to early globular stages. Of 662 ovules analyzed, 38.8% were mutant, 52.3% were wild type, and 8.9% were undeveloped.

[b] Refers to the total number of mutants analyzed ($N = 257$, last column).

[c] Frequency of numbers of endosperm nuclei arrested at a given stage correlated to embryo arrest.

[d] Ovule with embryo, no endosperm nuclei visible.

to produce viable seeds that were enlarged due to an enlargement of both embryo and endosperm (Scott *et al.* 1998). Whereas triploid embryos develop at the same rate as diploid embryos, tetraploid endosperms display an increased rate of endosperm nuclei proliferation (Scott *et al.* 1998). We also observed enlargement of embryo and endosperm in ovules of wild-type, *cap1/CAP1*, and *cap2/CAP2* plants fertilized with diploid pollen. In addition, 15–20% of the embryo sacs did not develop, regardless of the maternal genotype, presumably due to the genetic background of the tetraploid paternal line (Table 5). In both *cap1* and *cap2* embryo sacs, embryo and endosperm development could not be rescued by the presence of a supernumerary paternal

TABLE 4

***cap2* embryo and endosperm phenotype at different stages**

WT stage	Zygote	1 cell	2 cell	4 cell	8 cell	16 cell	>16 cell to globular	Developed ovule,[b] no visible embryo	*N*
A. Embryo phenotypes in WT and *cap2* in early stages[a] (%)									
Quadrant to octant									
Wild type	2	2	12	45	34	5	0	0	128
cap2	7	28	31	15	2	0	0	17	137
B. Embryo phenotypes in *cap2* in later stages[c,d] (%)									
Dermatogen stage									
cap2[c]	10	22	15	9	2	5	1	37	103
Globular/heart stage									
cap2[d]	1	1	3	2	1	4	30	57	236

WT embryo stage	No. of endosperm nuclei: 1	2–4	8–12	Multiple	Developed ovule,[b] no visible ESN	Developed ovule,[b] no visible ESN/embryo	*N*
C. Endosperm phenotypes in *cap2* correlated to embryo stages[e] (%)							
Quadrant to octant	11	28	13	2	30	17	136
Dermatogen	2	16	6	1	39	37	103
Globular/heart	0	5	2	14	22	57	236

Frequencies of *cap2* embryo and endosperm phenotypes in different WT stages. All frequencies are percentages of the mutant or WT class. Embryo and endosperm stages were determined by whole-mount clearing preparations of ovules from *cap2/CAP* females outcrossed with WT.

[a] Correlation of WT and *cap2* embryo development in quadrant to octant stage.

[b] Ovule was enlarged but endosperm and/or embryo degenerated.

[c] Phenotypical range of *cap2* embryo development in stages where WT is in the dermatogen stage.

[d] Range of *cap2* embryo phenoptyes in stages where WT is in the globular to heart stage.

[e] Range of *cap2* endosperm phenotypes correlated to WT embryo stages in the same silique. None of the mutant classes showed cellular endosperm. See text for details.

TABLE 5

Seed development in *capulet* in crosses with diploid, *MET1 a/s*, and *ddm1-2* pollen

Line	WT development (%)		Aborted embryo development (%)	*N*
	Developed embryo	Undeveloped embryo sac		
cap1/+ × TET(4C)	45.7	15.9	38.5	208
cap2/+ × TET(4C)	43.7	19.0	37.3	316
Ler × TET(4C)	78.5	17.7	3.8	368
cap1/+ × MET1/MET1 a/s	49.4	11.7	38.9	494
cap2/+ × MET1/MET1 a/s	48.9	12.1	39.0	577
Ler × MET1/MET1 a/s	90.9	7.7	1.4	439
fis1/fis1 × MET1/MET1 a/s	83.8	7.7	8.5	247
fis1/fis1 × fis1/fis1	1.4	7.3	91.3	427
cap1/+ × ddm1-2/ddm1-2	48.7	8.8	42.5	226
cap2/+ × ddm1-2/ddm1-2	50.0	8.1	41.9	346
Ler × × ddm1-2/ddm1-2	86.5	7.0	6.6	244
fis1/fis1 × × ddm1-2/ddm1-2	46.8	N/A	53.2	156

Embryo and endosperm phenotypes were determined by whole-mount clearing preparations of ovules. Embryo and endosperm stages were determined for each ovule. The female parent is listed first in all crosses presented. See RESULTS and MATERIALS AND METHODS for further details.

CAP allele (Table 5). *cap1/CAP1/CAP1* embryos were arrested as binucleate proembryos. *cap1/cap1/CAP1/CAP1* endosperms had somewhat enlarged nuclei but otherwise were phenotypically identical to *cap1/CAP1/CAP1* endosperms. The same result was obtained for *cap2* embryo sacs, although embryo and endosperm carrying a supernumerary *CAP2* allele seemed to degenerate prematurely. In summary, these data suggest that the mutant phenotypes of endosperm and embryo do not depend on their own *CAP* gene dosage but rather on the parental origin of the *cap* mutant allele.

No fertilization-independent seed development in *cap* mutant embryo sacs: In the maternal-effect mutants *mea* (also called *fis1*), *fis*, and *fie*, endosperm and, except in *fie*, embryo develop in the absence of fertilization (OHAD *et al.* 1996; CHAUDHURY *et al.* 1997; GROSSNIKLAUS *et al.* 1998). In our hands, 38% of *fis1* embryo sacs developed autonomously (see Table 6). To test whether *cap1* and *cap2* mutants also had this capability, we emasculated *cap/CAP* plants and analyzed ovules 5–10 days later. All embryo sacs were undeveloped in both mutant lines (Table 2), suggesting that, unlike *MEA* and related genes, *CAP1* and *CAP2* are not required for prefertilization repression of embryo and endosperm development.

***cap2* disrupts sexual and autonomous development of *fis1* mutant embryo sacs:** Disruption of *MEA*, *FIS*, and *FIE* genes leads to autonomous development of embryo and endosperm in unfertilized embryo sacs (OHAD *et al.* 1996; CHAUDHURY *et al.* 1997; GROSSNIKLAUS *et al.*

TABLE 6

Seed set and endosperm development in autonomous *fis1/FIS1;cap/CAP* double-mutant embryo sacs

Genotype	Frequency of autonomous seeds (%)	% endosperm nuclei phenotype in autonomous seeds[a]				*N*[e]
		Enlarged ESN[b]	Normal-sized ESN[c]			
		1–4[d]	1–4[d]	8–12[d]	>12[d]	
fis1/FIS;CAP/CAP, unpollinated	19	—	33	30	37	356
fis1/FIS;cap2/CAP2, unpollinated	19	49	14	16	21	340

Autonomous endosperm development was monitored by whole-mount clearing preparations of ovules from emasculated siliques 5–10 days after anther removal. See text for details. ESN, endosperm nuclei.

[a] Frequencies are calculated as percentage of autonomous seeds.

[b] Endosperm nuclear size in this class was in general enlarged and found only in autonomous seeds from *fis1/FIS1;cap/CAP* double mutants.

[c] Endosperm nuclear size is normal.

[d] Number of endosperm nuclei.

[e] Refers to both *fis1* and *FIS1* embryo sacs.

TABLE 7

Seed set in sexually developing *fis1/FIS1;cap2/CAP2* double mutants

Line	Seed phenotype[a] (%)			N^b
	WT torpedo stage	*fis1* arrested, heart stage	*cap2* arrested, degenerated small seed	
fis1/FIS selfed	50	50	0	247
fis1/FIS;cap2/CAP2 selfed	25	31	44	413
fis1/FIS;cap2/CAP2 × *Ler*	22	29	50	455

[a] Seed development was monitored by whole-mount clearing preparations of ovules. Seeds were scored for phenotype after the following criteria: WT seeds contained green torpedo to walking stick embryos and endosperm was cellularized; *fis1* seeds were white and contained early heart stage embryos; *cap2* seeds were developed, but arrested and degenerated.

[b] For the *fis1/FIS* selfed data three indpendent lines were used. For the *fis1/FIS;cap2/CAP2* selfed and WT outcross, two independent lines were used for each data set. The female parent is listed first in all crosses presented.

1998). To examine whether *CAP2* is required for development in *mea* mutant embryo sacs, we crossed plants homozygous for the *MEA* allele *fis1* (Luo *et al.* 1999) with *cap2/CAP2* pollen donors and analyzed their F_1 progeny for seed development (Tables 6 and 7). Four of nine F_1 plants were *fis1/FIS1* and exhibited normal seed set upon selfing, although embryo and endosperm development were arrested at heart stage and at endosperm cellularization, respectively, in approximately one-half of the seeds (Table 7). Five F_1 plants were classified as *fis1/FIS1;cap2/CAP2* because 50% of the seed showed the *cap2* phenotype. Furthermore, only one out of four seeds contained normal embryos upon selfing, as expected from genetic recombination between the *MEA* and *CAP2* loci at opposite ends of chromosome 1. Thus, *cap2* was epistatic to *fis1* in sexual seed development. This result was confirmed in crosses of *fis1/FIS1;cap2/CAP2* plants with wild-type pollen donors (Table 7).

To determine the effect of the *cap2* mutation on fertilization-independent seed development, we analyzed ovules of emasculated putative *fis1/FIS1;cap2/CAP2* and *fis1/FIS1;CAP2/CAP2* plants 5–10 days after anther removal (Table 6). For both genotypes, ~19% of the embryo sacs displayed autonomously developing endosperm (Table 6). In contrast to the *CAP2* control, 50% of the ovules from *fis1/FIS1;cap2/CAP2* contained endosperms with enlarged nuclei and were arrested after 0–2 syncytial mitoses (Table 6, Figure 4D). Some of the arrested endosperms were mononucleate or trinucleate, and, in rare cases, displayed mitotic spindles in metaphase (Figure 4C). These data indicate that *cap2* is epistatic to *fis1*, implying that *CAP2* is also required in autonomous seed development.

No rescue of the *cap* mutant phenotypes by altering epigenetic gene regulation: The maternal-effect genes *MEA*, *FIS2*, and *FIE* are expressed in an imprinted manner in early seed development (Kinoshita *et al.* 1999; Vielle-Calzada *et al.* 1999; Luo *et al.* 2000; Yadegari *et al.* 2000). In addition, their mutant phenotypes can be rescued by pollen genotypes, such as *METHYL TRANSFERASE1* antisense (*MET1 a/s*) transgene or mutations in *DECREASE IN DNA METHYLATION1* (*DDM1*), that are thought to alter epigenetic gene regulation (Kinoshita *et al.* 1999; Vielle-Calzada *et al.* 1999; Luo *et al.* 2000; Yadegari *et al.* 2000). We examined whether these pollen genotypes also rescued the maternal defects of *cap1* and *cap2* mutants. As a control, *fis1* mutant embryo sacs were fertilized with pollen carrying the *MET1 a/s* transgene (Table 5). Ovules from the pollinated siliques were examined from 1 to 8 DAP. The control *fis1* embryo sacs produced 84 and 47% viable seeds in the presence of *MET1 a/s* and *ddm1-2*, respectively (Table 5). By contrast, *cap1* and *cap2* embryo sacs displayed their characteristic developmental defects, regardless of the presence or absence of *MET1 a/s* and *ddm1-2* (Table 5). The developing endosperms from *CAP1* and *CAP2* sister embryo sacs carrying the *MET1 a/s* transgene had larger but fewer endosperm nuclei than those usually found after outcrosses to wild type, which is consistent with the findings of Adams *et al.* (2000).

***FIS2::GUS* expression in *cap* mutant endosperm:** To further investigate the roles of the *CAP* genes in endosperm development, we analyzed the expression of a *FIS2::GUS* transgene in the *cap* mutants (Luo *et al.* 2000). *FIS2* encodes a C_2H_2 zinc-finger transcription factor proposed to indirectly regulate the maternal-effect genes *FIE* and *MEA* (Luo *et al.* 1999, 2000). Due to imprinting of *FIS2*, the *FIS2::GUS* transgene is not expressed in the endosperm when introduced via pollen (Luo *et al.* 2000). When wild-type, *cap1/CAP1*, or *cap2/CAP2* plants were crossed with *FIS2::GUS* transgenic donors, no GUS expression was observed during endosperm development, indicating that *cap1* and *cap2* mutations did not relieve imprinting-mediated expression barriers.

To study *FIS2::GUS* expression in *cap1* and *cap2* mutant embryo sacs, *FIS2::GUS* transgenic plants were crossed with *cap1/CAP1*, *cap2/CAP2*, and wild-type con-

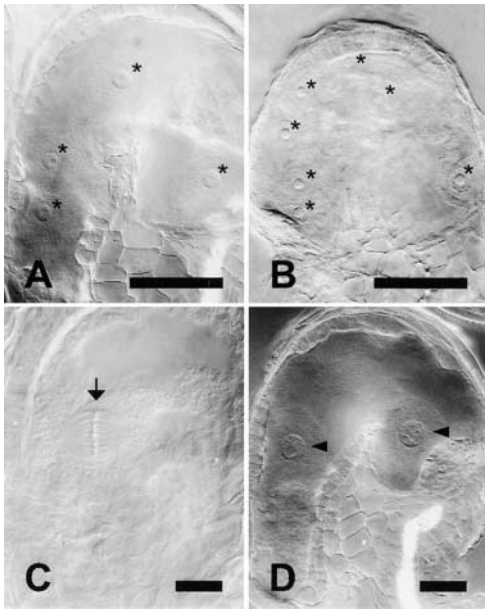

FIGURE 4.—*cap2 fis1* autonomous endosperm development. (A and B) *fis1* control. Several endosperm nuclei (asterisks) are shown. (C and D) *fis1 cap2*: arrested metaphase (C, arrow) and arrested enlarged endosperm nuclei (D, arrowheads) are shown. Optical sections were obtained from whole-mount preparations of unfertilized ovules 5–10 days after anther removal. Bars: A and B, 20 μm; C and D, 10 μm.

trol pollen donors. F_1 plants were emasculated and pollinated with wild-type pollen, and ovules were stained for GUS activity. The wild-type control yielded the expected temporal and spatial expression pattern in the developing endosperm (Figure 5, A–F; see also LUO *et al.* 2000). GUS expression was initially observed in the polar nuclei before and after fusion (Figure 5, A and B). After fertilization, GUS expression was strictly limited to endosperm nuclei during the first five to six syncytial mitoses (Figure 5, C–E) before GUS expression became restricted to the nuclear cyst of the CZE (Figure 5F).

Ovules from *cap1/CAP1;FIS2::GUS/−* plants gave the same overall staining frequency as *CAP1* controls, and 45% (N = 121) of the *cap1* mutant embryo sacs expressed the *FIS2::GUS* transgene in the endosperm. The GUS signal was present in all endosperm nuclei, although it was much weaker than that in the *CAP1* control (compare Figure 5G to Figure 5D), decreasing with each syncytial mitosis. The weaker staining may reflect lower expression levels of *FIS2::GUS* in the *cap1* background or, alternatively, expression of the transgene may have ceased early, with the remaining protein being partitioned during each free nuclear division.

cap2 mutant endosperms displayed high levels of *FIS2::GUS* expression in their enlarged nuclei at 3 DAP (Figure 5, H and I). At 5–7 DAP, *CAP2* endosperms expressed the transgene exclusively in the chalazal cyst (Figure 5F) that is missing in *cap2* endosperms (see above). Instead, nuclear *FIS2::GUS* expression persisted

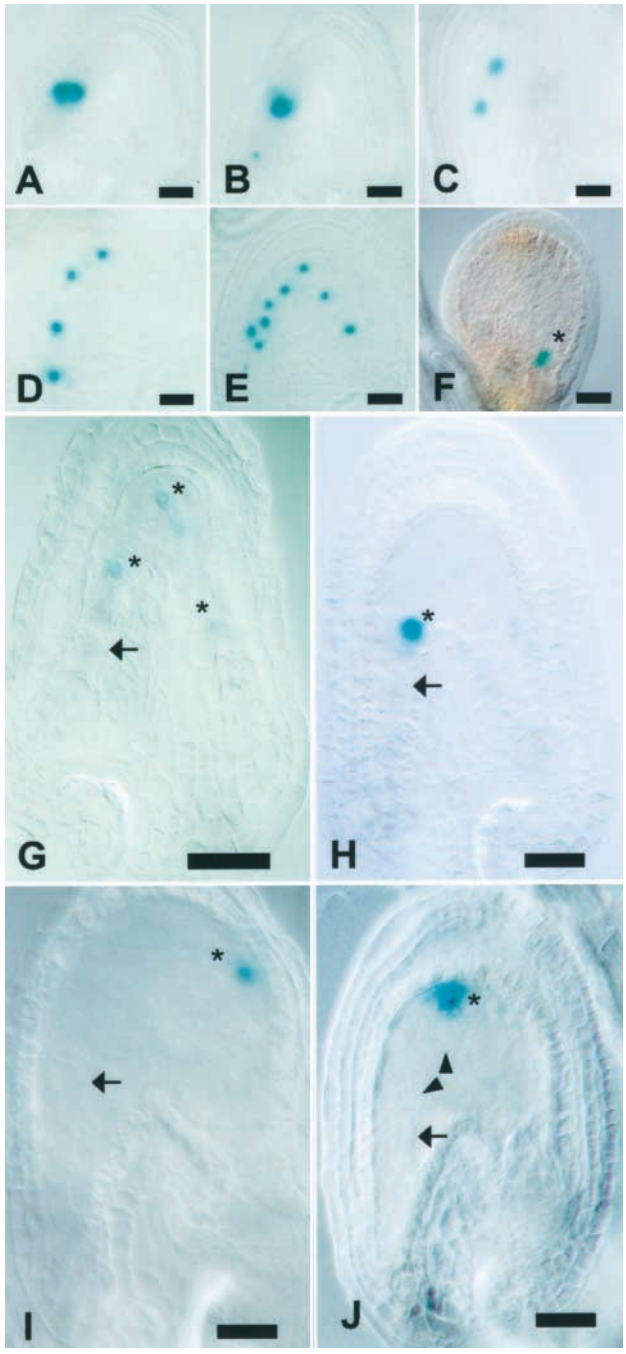

FIGURE 5.—*FIS2::GUS* expression in *cap1* and *cap2* endosperm. (A–F) Wild-type control. GUS expression starts in central cell nuclei before fusion (A) and continues after fusion (B) and in syncytial endosperm nuclei (C–E) and is later restricted to the chalazal cyst (F, asterisk). (G) *cap1*: faint GUS staining in endosperm nuclei (asterisks). (H–J) *cap2*: strong GUS staining in enlarged endosperm nuclei (asterisks). Note lack of GUS staining in some endosperm nuclei (J, arrowheads). Optical sections were obtained from whole-mount preparations of GUS-stained ovules. Arrows, embryos. Bars: A–E, 10 μm; F–J, 20 μm.

in the peripheral and micropylar regions of *cap2* endosperms (Figure 5, H–J) whereas other nuclei lacked detectable GUS activity (Figure 5J, arrowheads). Thus,

cap1 and *cap2* mutant embryo sacs were able to activate the *FIS2::GUS* transgene.

DISCUSSION

We have isolated two gametophytic mutants, *cap1* and *cap2*, in which both embryo and endosperm are developmentally arrested only if the female gametophyte carries the mutant alleles. Our linkage-based screen ruled out both incompletely penetrant dominant maternal effects of the sporophyte and incompletely penetrant dominant zygotic mutations as the mutant phenotype that is always segregated with the genotype of the female gametophyte during meiotic recombination. We will first discuss whether this apparent gametophytic maternal effect is caused by the mutant alleles in the female gametophyte itself or instead by the inactivity of the pollen-derived wild-type alleles during embryo and endosperm development. Subsequently, we will address possible roles of the *CAP* gene functions in embryo and endosperm development.

Gametophytic maternal effect or gamete-specific imprinting of *CAP* genes? By genetic criteria, the *CAP* genotype of the haploid embryo sac determined whether or not its fertilization products, the diploid embryo and the triploid endosperm, developed normally. The *CAP* genotype of the pollen had no effect, as shown by reciprocal crosses between *cap/CAP* heterozygous plants and wild-type plants as well as by crosses between *cap/CAP* plants and tetraploid pollen donor plants. Furthermore, the *CAP2* gene was also required in the autonomous development of *fis1* diploid endosperm from unfertilized embryo sacs.

A 2:1 ratio of maternal-to-paternal genomes has long been recognized as crucial for proper endosperm development (for review, see Scott *et al.* 1998). Parents of the same ploidy produce viable seed, whereas reciprocal interploidy crosses lead to a reduction or increase in seed size or, in extreme cases, to seed abortion (Lin 1984; Birchler 1993; Scott *et al.* 1998). Different mechanisms have been proposed to account for this effect. The parental conflict theory suggests that conflicting interests of the maternal and paternal genomes are balanced by genomic imprinting (Lin 1984; Haig and Westoby 1989; Scott *et al.* 1998). Experimental evidence for imprinting comes from preferential expression of maternal *zein* alleles in maize (Lund *et al.* 1995) as well as from recent analyses of the Arabidopsis *MEA*, *FIS*, and *FIE* genes (Kinoshita *et al.* 1999; Vielle-Calzada *et al.* 1999, 2000; Luo *et al.* 2000; Vinkenoog *et al.* 2000; Yadegari *et al.* 2000). The critical question is whether the *cap* mutant embryo and endosperm phenotypes are due to imprinting of the paternal alleles or due to a true maternal effect of the female gametophyte.

Mutants of the *MEA*, *FIS*, and *FIE* genes can be rescued by the *ddm1* mutation or by *MET1 a/s* transgene expression, which supports the notion that these genes are imprinted in the paternal genome (Kinoshita *et al.* 1999; Vielle-Calzada *et al.* 1999; Luo *et al.* 2000; Vinkenoog *et al.* 2000). Using the same approach, *cap1* and *cap2* mutants were not rescued, suggesting that the *CAP* genes are not imprinted in a similar way. However, *DDM1* and *MET1* may not be involved in all sorts of genomic imprinting. *MET1* is the major maintenance cytosine methyltransferase in Arabidopsis, and a functional knockout by *METI a/s* transgene expression reduces methylation to 15% of the wild-type level (Finnegan and Dennis 1993; Finnegan *et al.* 1996; Genger *et al.* 1999). The *DDM1* gene product is a member of the SWI2/SNF2 protein family of chromatin remodeling factors (Jeddeloh *et al.* 1999), and its primary effect in imprinting may involve changes in chromatin conformation (Vongs *et al.* 1993; Kakutani *et al.* 1995, 1996).

The parent-specific effects in *mea* and *fie* mutants may involve different imprinting mechanisms, since *ddm1* pollen rescues *mea* mutants but not *fie* mutants (Yadegari *et al.* 2000). In addition, rescue by the *MET1 a/s* transgene also occurs when the pollen is mutant for *mea* and *fis* but not for *fie*, thus indicating that the phenotypical rescue is not mediated by the activation of paternal alleles and suggesting that different factors are involved in relieving transcriptional silencing (Luo *et al.* 2000; Vinkenoog *et al.* 2000). Candidates include genes such as *HOG1*, *MOM1*, *SIL1*, *SIL2*, and *SOM/DDM*, which modify transcriptional gene silencing in Arabidopsis (Vongs *et al.* 1993; Furner *et al.* 1998; Mittelsten Scheid *et al.* 1998; Amedeo *et al.* 2000). Considering that mechanisms for imprinting and transcriptional gene silencing are not fully understood in Arabidopsis, we cannot rule out imprinting of *CAP1* and *CAP2* genes. However, so far no evidence supports imprinting of these genes and therefore we consider it more likely that *cap1* and *cap2* mutants represent true gametophytic maternal-effect mutants.

Possible roles of *CAP* gene functions in embryo and endosperm development: The development of *cap1* embryos was arrested very early, with no mutant embryos progressing beyond a binucleate proembryo stage. Furthermore, most embryos were arrested before this stage, suggesting that *CAP1* gene function is required from fertilization on in the developing embryo itself. By contrast, the developmental arrest of *cap2* embryos was delayed, and in rare cases, the embryo developed to the early heart stage. *cap2* embryos displayed no major pattern defects although they appeared enlarged compared to equivalent wild-type stages. The later developmental arrest of *cap2* embryos could result from a nursing defect of the mutant endosperm (see also review by Lopes and Larkins 1993). In this interpretation, the primary target of *CAP2* function would be the developing endosperm.

Both *cap1* and *cap2* affect endosperm development, although in different ways. In the *cap1* endosperm, development is arrested after a few syncytial mitotic divi-

sions, thus resembling the defect observed in the *cap1* embryo. It is therefore likely that *CAP1* gene function is required independently in both embryo and endosperm development, in contrast to *CAP2*. It is conceivable, for example, that *CAP1* plays an activating role after fertilization and that, in its absence, embryo and endosperm development depend on maternal supplies. This idea is supported by the decreasing *FIS2::GUS* signal in *cap1* endosperms, which may result from prefertilization expression in the central cell. The presence of maternal supplies in early embryo and endosperm development is also evidenced by one or a few mitotic divisions that occur after fertilization in the absence of zygotic factors required for the formation and/or maintenance of the microtubular cytoskeleton (Mayer *et al.* 1999). In contrast to *cap1*, *cap2* endosperm nuclei are irregular in shape and size, possibly representing variable endoreduplication cycles. The *FIS2::GUS* expression data also suggest regional heterogeneity among endosperm nuclei. Whether this reflects interference with the cell division machinery or incorrect specification of endosperm domains remains to be determined.

The *cap* mutants represent novel genes that are presumably expressed in the female gametophyte itself and whose products are required for embryo and endosperm development. This interpretation can be rigorously tested only by the molecular characterization of the *CAP* genes, which allows the molecular basis for their gametophytic maternal influence on seed development to be determined. The *CAP* genes may thus represent models for the analysis of maternal factors and mechanisms crucial for embryo and endosperm development.

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Note added in proof: While this manuscript was under review, Choi *et al.* (Y. Choi, M. Gehring, L. Johnson, M. Hannon, J. J. Harada *et al.*, 2002, DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in Arabidopsis. Cell **110:** 33–42) provided evidence that activation of the maternal allele of *MEDEA* in the central cell depends on the activity of *DEMETER*.

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