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The *mac1* Gene: Controlling the Commitment to the Meiotic Pathway in Maize

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Manuscript received September 5, 1995
Accepted for publication December 1, 1995

ABSTRACT

The switch from the vegetative to the reproductive pathway of development in flowering plants requires the commitment of the subepidermal cells of the ovules and anthers to enter the meiotic pathway. These cells, the hypodermal cells, either directly or indirectly form the archesporial cells that, in turn, differentiate into the megasporocytes and microsporocytes. We have isolated a recessive pleiotropic mutation that we have termed *multiple archesporial cells1* (*mac1*) and located it to the short arm of chromosome *10*. Its cytological phenotype suggests that this locus plays an important role in the switch of the hypodermal cells from the vegetative to the meiotic (sporogenous) pathway in maize ovules. During normal ovule development in maize, only a single hypodermal cell develops into an archesporial cell and this differentiates into the single megasporocyte. In *mac1* mutant ovules several hypodermal cells develop into archesporial cells, and the resulting megasporocytes undergo a normal meiosis. More than one megaspore survives in the tetrad and more than one embryo sac is formed in each ovule. Ears on mutant plants show partial sterility resulting from abnormalities in megaspore differentiation and embryo sac formation. The sporophytic expression of this gene is therefore also important for normal female gametophyte development.

HIGHER plant megasporogenesis, megagametogenesis and embryogenesis have been studied intensively during the past century. Light and electron microscopy studies have provided detailed descriptions of the morphological and anatomical changes that characterize these processes (RANDOLPH 1936; KIESSELBACH 1949; MAHESHWARI 1950). Recent techniques for the isolation of both the megaspore mother cells (MMCs) and the embryo-sac combined with immunofluorescent staining of tubulin and DNA staining have provided data about cellular polarity during megasporogenesis and megagametogenesis and the role of the polarity in the fate of the nuclei during these processes (HUANG and RUSSELL 1989, 1993; REISER and FISCHER 1993; WEST and HARADA 1993; HUANG and SHERIDAN 1994). Recent research in both sperm and egg biology have been fruitful for understanding the role of these cells in the process of double fertilization. During the past few years it has become possible with some angiosperm species to isolate sperm, eggs and central cells and to offer new approaches for studying *in vitro* fertilization in higher plants (HUANG and RUSSELL 1992; DUMAS and MOGENSEN 1993; KRANZ and LORZ 1993; BEDINGER and RUSSELL 1994; HOLM *et al.* 1994, 1995).

The genetic control of commitment to the meiotic pathway: In contrast with the progress in studying the cell biology of gametogenesis little is known about the initial events in the development of the premeiotic sporogenous cells and especially about the genes that control the formation of the archesporium in the ovules and anthers of angiosperms. It is of fundamental interest to understand how the hypodermal cell becomes committed to the meiotic pathway, how this commitment is limited to but a single cell in an ovule, and what is the genetic program underlying and regulating the cellular changes that occur as the archesporial cell develops into the sporocyte.

The origin of meiotic cells in flowering plants: In angiosperms meiosis takes place in the female sporangium, within the ovule, and in the male sporangium, within the anther. During the early development of both the ovule and the anther, one or more of the cells located just below the epidermal layer, the hypodermal cells, becomes clearly distinguished from the rest of the nucellar cells because of their larger size and their location. In most flowering plants only one hypodermal cell gives rise to an archesporial cell in each ovule, and this cell differentiates directly into the megasporocyte (megaspore mother cell, MMC) without any intervening mitotic cell division (MAHESHWARI 1950; GIFFORD and FOSTER 1987; REISER and FISCHER 1993). Although most angiosperms have but a single archesporial cell and a resulting single megasporocyte cell per ovule, some taxa form multiple megasporocytes. These include *Paeonia californica,* which may form 30–40 megasporocytes, many of which undergo a normal meiosis to form a linear tetrad of megaspores, while others de-

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generate before entering meiosis (WALTERS 1962). Such a large number is an exceptional case, but multiple megasporoctyes are found in the Casuarinaceae and some members of the Amentiferae and Ranales (EAMES 1961). Two embryo sacs per ovule have been reported amongst a wide range of primitive genera of the dicotyledons, including members of the Rosaceae and the Ranunculaceae as well as among genera in the Compositae (COULTER and CHAMBERLAIN 1903; MAHESHWARI 1950).

The origin of meiotic cells in maize ovules: In the pseudocrassinucellate type ovule of maize a single hypodermal cell develops directly into an archesporial cell that, without any intervening mitotic divisions, differentiates into the megasporocyte (DAVIS 1966). Subsequently, the meiocyte becomes more deeply embedded in the nucellus of the ovule as a result of divisions in the overlying epidermal cell layer that produce subepidermal rows of parietal-like cells (RANDOLPH 1936; COOPER 1937; KIESSELBACH 1949). The hypodermal cell enlarges as it develops into the archesporial cell. This cell enlargement and accumulation of cellular organelles to form a dense cytoplasm continues as the archesporial cell differentiates into the megasporocyte. During this process the meiocyte elongates and becomes tear-drop or pear-shaped with its micropylar end widening (HUANG and RUSSELL 1992; RUSSELL 1993).

We are especially interested in learning how genes control the alternation between the diploid sporophyte generation and the haploid gametophyte generation, the sequence of the events leading to meiosis and the development of the female reproductive system. We aim to identify and characterize the maize genes that are specifically involved in regulating meiosis and female gametophyte development. Here we present the result of a mutational analysis that identifies one step in the genetic control of the shift from the somatic cell pathway into the meiotic pathway of maize. In an earlier paper (GOLUBOVSKAYA *et al.* 1993) we reported that in mutant plants the microsporocytes are blocked at prophase I of meiosis, and we referred to this mutant by the laboratory designation *leptotene arrest*-487* (*lar*-487*). We now report that in each mutant ovule several hypodermal cells become committed to the meiotic pathway, instead of the single hypodermal cell that proceeds toward meiosis in a normal ovule. This newly identified recessive sporophytic mutation appears to control the development of the hypodermal cell into the archesporial cell, and it appears also to be important for female gametophyte development.

MATERIALS AND METHODS

The new mutant was isolated from an active Robertson's Mutator stock while screening for male sterile mutants. The mutant was previously shown to be inherited as monogenic recessive mutation (GOLUBOVSKAYA *et al.* 1993). We now designate the gene identified by this mutation as *multiple archesporial cells1* (*mac1*). Families of plants segregating in a 3:1 ratio for fertile plants with normal meiosis and male sterile plants with the mac1 cytological phenotype were used in this study.

Genetic analysis: To map the *mac1* recessive gene to chromosome arm location, the fertile plants from families that segregated for *mac1* were crossed as a female parent by the basic set of B-A translocation stocks (BECKETT 1994). The resulting F_1 progenies were analyzed for segregation for male sterility. The *waxy1* translocation stock *wx1 T9–10b* (*9S.13 10S.4*) was crossed as a male onto *mac1/mac1* ears. The resulting F_1 progeny were self-pollinated to produce F_2 progeny. Linkage of *mac1* with *wx1* was analyzed by the product method of IMMER (1930) for alleles in repulsion (see Appendix 2 in REDEI 1982). Because the waxy trait displays pseudolinkage with the translocation breakpoint at *10S.4* (ANDERSON 1956), this analysis can yield information regarding the linkage between *mac1* and the breakpoint on *10S*.

Cytology of the *mac1* mutant: For the cytological examination of microsporocytes, immature tassels were taken from fertile and male sterile sibling plants and fixed in Farmer's (three parts ethanol: one part glacial acetic acid) fixative. For cytological examination of megasporoctyes, the immature ears from five normal plants and from five mutant sibling plants were fixed for 24 hr at room temperature in FAA fixative (40% formaldehyde; glacial acetic acid: 50% ethanol in a 5:5:90 volume ratio). After 14 hr in 95% ethanol, the fixed samples were stored in 70% ethanol at 4° before analysis. Microphotography was carried out with microcamera MFN 11 using a Biolar microscope (see GOLUBOVSKAYA *et al.* 1992, for details of the fixation, dissection and squash techniques of the isolated megasporocytes.) Mature embryo sacs were dissected following enzyme digestion from normal and mutant ovules with glass needles, and their nuclei stained with DAPI (4, 6 diamidine-2-phenylindole dihydrochloride; Sigma) according to the procedures of HUANG and SHERIDAN (1994).

Ovule anatomy: For the anatomical study of the *mac1* mutant megasporogenesis, the ovules of the mutant plants were fixed in FAA, embedded in paraffin and sectioned with a microtome. Three stains were used: the Feulgen reagent for nuclear staining, hematoxylin for staining of the cell cytoplasm, and Alcian blue for cell wall staining. Anatomical study was performed with an Amplival Zeiss microscope, and the drawings were carried out with aid of a camera lucida with (100 × 7) magnification. For plastic sectioning some mutant and normal ovules were fixed in 3% glutaraldehyde, postfixed in 1% osmium textroxide, embedded in Spurr's resin and sectioned at 4-micron thickness as described in HUANG and SHERIDAN (1994).

RESULTS

The *mac1* locus is not uncovered by TB-10L19 but is closely linked to its breakpoint: Several male plants were crossed by *TB10-L19* with the goal of testing for the location of the *mac1* locus on chromosome arm *10L*. Large families of 75 or more kernels were planted from five ears produced by crossing with *TB-10L19* pollen. None of the progeny grown from these five ears segregated for male sterility. This result indicated that the *mac1* locus either was not located on *10L* or if it were, then the locus was proximal to the breakpoint on this arm. Hypoploid plants (carrying a normal chromosome *10* and a *10L-B* chromosome but no *B-10L* chromosome) were identified amongst the *TB-10L19* progeny by the presence of 50% pollen sterility and several plants were self-pollinated. Progeny were grown from four self-pollinated ears of hypoploid plants, and their

pooled scoring results were seven fertile plants and 65 male sterile plants. It was evident that the normal chromosome *10* carried the *mac1* mutant allele and that the fertile plants were the result of a crossover having occurred between that locus and the breakpoint located at cytological position ~0.01 distal to the centromere on the *10L-B* chromosome. Because only two of the four theoretically possible phenotype classes were recovered amongst these F_2 progeny (the *10L-B* chromosome did not transmit), the product method could not be applied to calculate linkage. However, because the parental type (*mac1/mac1*) was recovered with about a 90% frequency, the parental type gamete frequency can be estimated by $\sqrt{0.90} = 0.95$. This value indicates a recombination frequency of ~5% between the breakpoint on *10L* and the *mac1* locus. This result suggested that the *mac1* locus is located in the proximal region of chromosome arm *10S*.

The *mac1* locus is on chromosome arm *10S*: Crosses of six *mac1/mac1* plants by *TB10-Sc* resulted in a pooled progeny of 41 plants that segregated 29 fertile plants and 12 male sterile plants thereby confirming the location of the *mac1* locus on chromosome arm *10S*. The F_2 progeny of the cross of *wx1 T9–10b* onto *mac1* plants segregated 67 starchy fertile, 34 starchy male sterile, 88 waxy fertile and three waxy sterile progeny. Analysis of these results by the product method resulted in a recombination value between *mac1* and *wx1* of 17.6 with a standard error of ±0.07. The breakpoint for *TB-10Sc* is at a position 0.3 distal to the centromere on *10S*, consequently *mac1* must be distal to 0.3 on *10S*. The breakpoint for *Twx 9–10b* is at a position 0.4 distal to the centromere on *10S*. The 17.6% recombination between *mac1* and *wx1* is a reflection of crossing over between the breakpoint at 0.13 on *9S* and the *wx1* locus on *9S* as well as crossing over between the breakpoint at 0.4 on *10S* and the *mac1* locus (regardless of whether the *mac1* locus is distal or proximal to 0.4 on *10S*). Because ANDERSON (1938) reported that the recombination between the *waxy1* locus on *9S* and the *9S* breakpoint in the *9–10b* translocation was 5.7%, about two-thirds or more of the 17.6% recombination we observed between *waxy* and *mac1* would be expected to occur between *mac1* and the breakpoint at 0.4 on *10S*. Therefore it is likely that the *mac1* locus is located at a position distal to 0.4 on chromosome arm *10S*. Inasmuch as none of the named meiotic mutations of maize are located on *10S* or display this mutant phenotype, we have assigned the *multiple archesporial cells1* (*mac1*) gene symbol to this locus.

Microsporogenesis in normal and *mac1* mutant plants: We have previously reported that the fertile siblings of families segregating for *mac1* have a normal set of meiotic divisions while homozygous mutant (*mac1/mac1*) plants are male sterile and defective in their male meiosis with the mutant microsporocytes arresting at the leptotene stage of meiotic prophase I (GOLUBOVSKAYA *et al.* 1993). At the time of that report we had not examined female meiosis in *mac1* megasporocytes, although we had observed that mutant *mac1* plants were completely male sterile and partially female fertile.

Megasporogenesis in normal siblings: From four fertile plants, M6808–2, -4, -5, and -31, a total of 20, 21, 15, and 14 ovules were isolated, respectively. Cytological analyses of these 70 ovules revealed that in every case each ovule contained a single MMC or megagametophyte. Amongst the group of ovules from each of the four plants, a sequence of normal meiotic stages and normal postmeiotic mitotic divisions were observed [see text and Figure 1 of GOLUBOVSKAYA *et al.* (1992) for a description of normal female meiosis in maize]. In summary, the normal ovules contained but a single MMC. This cell underwent a normal meiosis that frequently resulted in the formation of a linear tetrad of megaspores, only the lower (chalazal-most) megaspore survived and developed into a normal eight-nucleate embryo sac.

Megasporogenesis in *mac1* siblings: A total of 63 individual ovules from three male sterile *(mac1/mac1)* mutant plants were cytologically analyzed. The results of the analyses for each individual ovule are presented in the Table A1 in the APPENDIX and in summary form in Table 1. Four interesting features of these data may be noted.

There were multiple MMCs in each of the 63 mutant ovules examined: The 26 ovules of plant M6808–30 contained 230 sporogenous cells, the 30 ovules of plant 6734–6 contained 303 sporogenous cells and the seven ovules of plant 6808–6 contained 47 sporogenous cells (Table 1). Together these 63 ovules contained a total of 580 sporogenous cells, ranging from 3 to 21 sporogenous cells per ovule (Table A1, APPENDIX) with a mean of 9.3 sporogenous cells per ovule, a SD of 3.8 and a SE of 0.5. About 65% of the mutant ovules contained between six and 11 sporogenous cells per ovule, and nearly 86% of the ovules contained between four and 13 sporogenous cells per ovule (Table A1, APPENDIX).

The multiple MMCs in each mutant ovule developed from archesporial cells derived directly from multiple hypodermal cells: Sectioning of normal ovules early in their development revealed the expected single archesporial cell per ovule (Figure 1a). In contrast sectioning of mutant ovules revealed a single layer of several enlarged hypodermal cells that were developing into archesporial cells (Figures 1b and 2, A and B). Each hypodermal cell appears to develop directly without any intervening mitotic division into an archesporial cell and only rarely was a hypodermal cell observed to undergo a periclinal division to produce two daughter cells (Figure 2C). The archesporial cells were observed to subsequently enlarge into MMCs and enter prophase I of meiosis (Figure 2D). Sectioning of mutant ovules at a later stage of development revealed the presence of several MMCs at the dyad stage of meiosis or at the two or four nucleate stage of embryo sac formation (Figure 3A). Additional sections revealed within a single ovule the

TABLE 1

Summary of stage distribution of MMCs and embryo sacs (ESs) in normal and mutant ovules

No. of analyzed plants	Number of ovules analyzed	Stages of meiotic division[a]										Embryo sac stage: No. of nuclei			Total[b] number of MMCs and ESs
		Premeiosis	L.	Z	P	Dip	Dia	MI	A-TI	Dy	Tet	2	4	8	
Normal															
6808-2	20	0	0	5	1	0	1	0	0	2	2* 5	1	3	0	20
6808-4	21	0	0	2	2	0	0	2	1	1	10	1	0	2	21
6808-5	15	0	0	2	3	0	0	0	1	0	1* 2	4	1	1	15
6808-31	14	0	0	3	1	0	2	0	0	2	0* 2	3	1	0	14
Total	70	0	0	12	7	0	3	2	2	5	3* 19	9	5	3	70
Mutant															
6808-30	26	18	10	43	22	1	6	5	2	4	58	27	28	6	230
6734-6	30	124	0	79	27	8	11	3	5	10	32	2	1	1	303
6808-6	7	0	0	9	5	0	1	1	3	2	10	7	7	2	47
Total	63	142	10	131	54	9	18	9	10	16	100	36	36	9	580
%		24.5	1.7	22.6	9.3	1.6	3.1	1.6	1.7	2.7	17.2	6.2	6.2	1.6	100

[a] Abbreviations of meiotic stages: L, leptotene; Z, zygotene; P, pachytene; Dip, diplotene; Dia, diakinesis; *, M-T2; Mi, metaphase I; A-TI, anaphase-telophase I; Dy, dyad; Tet, tetrad.

[b] Note that for each normal plant the total number of MMCs and ESs equals the total number of ovules analyzed because each ovule contained only one MMC or ES. See Table A1 in the APPENDIX for detailed data for each normal and mutant ovule.

presence of MMCs at the dyad stage (Figure 3B) and tetrad stages containing four megaspores in a quadrant and also in the usual linear arrangement as well as two and four nucleate embryo sacs (Figure 3, C and D).

The sporogenous cells were not synchronized in their development: The sporogenous cells of an individual ovule always displayed a range of developmental stages that, depending on the ovule, could include the premeiotic stage, meiotic stages, and the two-, four- and eight-nucleate stages of embryo sac development. Examples of this are shown in Figure 3. The data of the APPENDIX Table A1 presents the detailed cytological examination of each of the 63 mutant ovules. An example of what was observed in these microscopic analyses is presented in Figure 4. Figure 4a presents the contents of a single mutant ovule; the 18 sporogenous cells

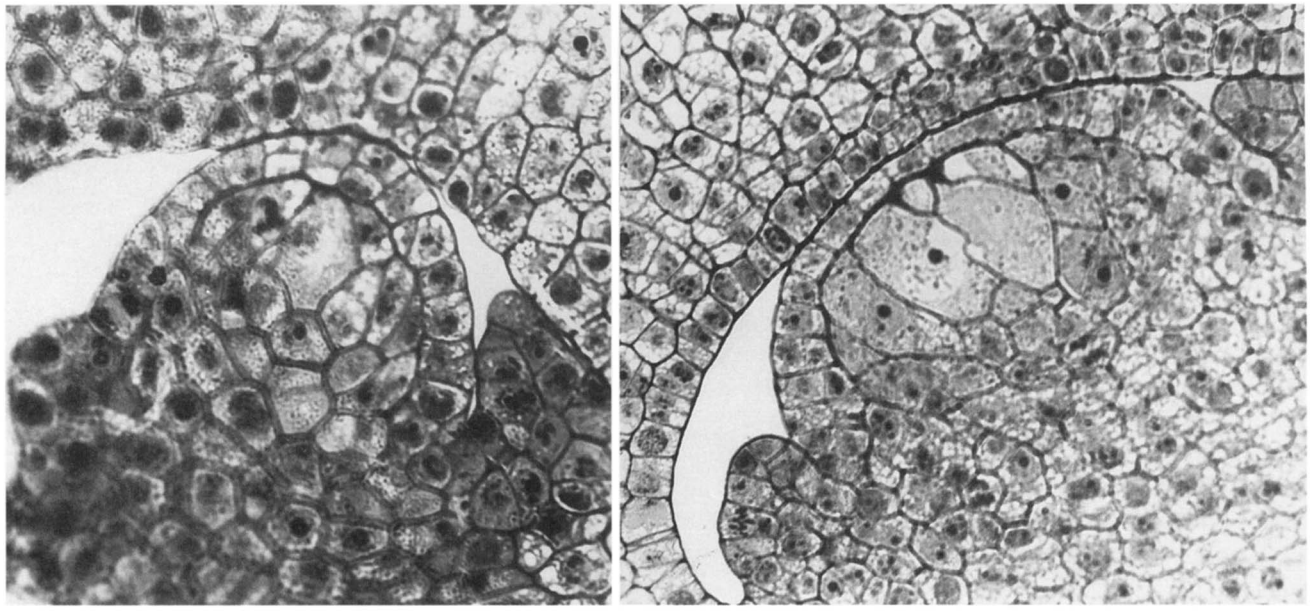

FIGURE 1.—Anatomy of normal and *mac1* mutant ovules. (a and b) Longitudinal plastic section stained with Toluidine Blue. (a) Sectioned ovule of normal plant: only one megaspore mother cell is present per ovule and it is located just under the epidermal layer. (b) Sectioned ovule of *mac1* homozygous plant; several megaspore mother cells are present per ovule and at least six of them are clearly seen in this section. All are located under the epidermal layer as in the normal plant.

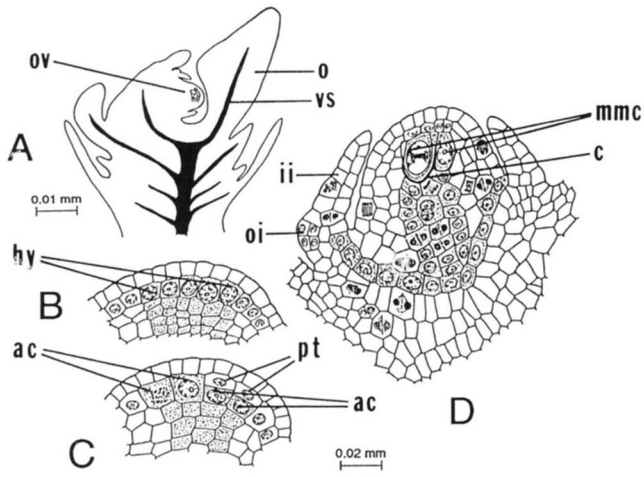

FIGURE 2.—Section of paraffin-embedded ovary at an early stage of development in the *mac1* mutant. Drawing with camera lucida, magnification is shown. (A) View of longitudinal sectioned ovary, ovule position is shown. (B–D) Sporogenous complex formation; development of several archesporial cells is shown. (B) Enlarged hypodermal cells are located directly under nucellar epidermis. (C) A periclinal division of two hypodermal cells and formation of archesporial cells and parietal-like cells. (D) View of ovule: from top to bottom—a layer of nucellar epidermis and two megaspore mother cells at the prophase I stages. o, ovary; ov, ovule; ac, archesporial cell; MMC, megaspore mother cell; hy, hypodermal cell; vs, vascular strand; ii, inner integument; oi, outer integument; pt, parietal-like cell; c, callose.

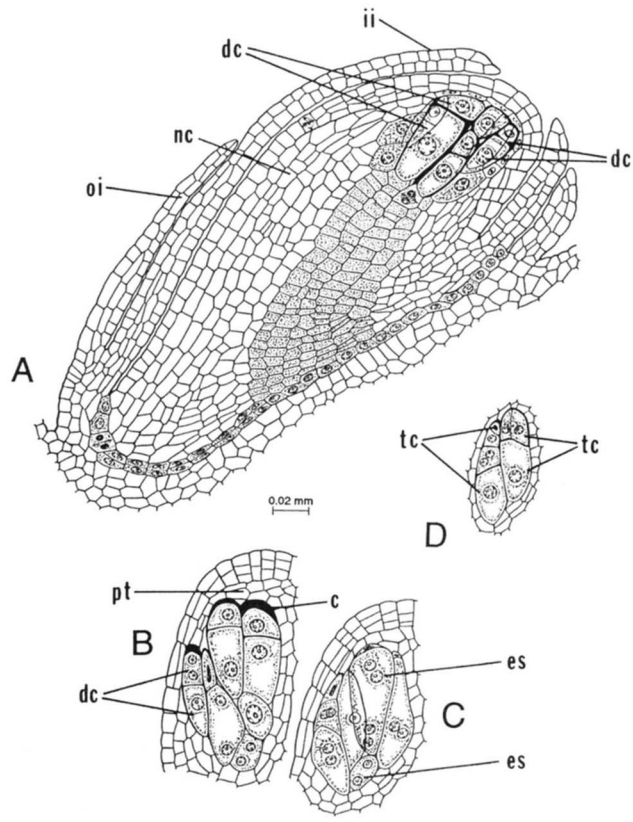

FIGURE 3.—The ovule at a late stage of development in the *mac1* mutant. Drawing with camera lucida, magnification is shown. (A) Several MMCs at the different stages of meiosis. (B–D) Three serial sections through the same ovule: MMCs at the dyad stage (B), two- and four-nucleate embryo-sacs (C), MMCs at the tetrad stage of meiosis, T-shaped tetrad is clearly shown (D). ii, inner integument; oi, outer integument; nc, nucellar cell; pt, parietal-like cell; dc, MMC at the dyad stage; tc, MMC at the tetrad stage; es, embryo-sac; c, callose.

ranged from a cluster of six archesporial cells, through three premeiotic MMCs, five MMCs at the zygotene stage, one at the diplotene stage, two at the anaphase I stage and one at the telophase I stage. Figure 4b presents seven sporogenous structures ranging from a premeiotic MMC through the tetrad stage of meiosis containing four megaspores.

Another example of range of developmental stage of the sporogenous cells of an individual mutant ovule is presented in Figure 5a where eight sporogenous cells or cell groups of ovule No. 6 of plant M6808–30 are depicted. Three of the cells were in meiotic prophase I, one at metaphase I, two at the tetrad stage, one at the two-celled embryos sac stage and one at the four-cells embryo sac stage (Figure 5, b–i). Additional two- and four-nucleate embryo sac stages were observed for this ovule but they are not included in Figure 5.

Each mutant ovule appeared to contain a limited pool of MMC precursor cells: In each mutant ovule the group of enlarged hypodermal cells developed via archesporial cells into MMCs. All of these MMCs proceeded through a normal meiosis I and meiosis II. Many of the ovules contained developing embryo sacs. A summary of the data for the distribution of stages of development of the sporogenous cells of the 63 mutant ovules is presented in Table 2. Amongst the 63 ovules analyzed none of them contained sporogenous cells that were all in the premeiotic stage. Although five ovules each contained sporogenous cells that ranged from the premeiotic stage through embryo sac development, the other 58 ovules contained sporogenous cells spanning only a portion of the developmental stages over the range from premeiosis to embryo sac development. Taken as a whole, these data indicate that the mutant ovules did not contain a cohort of "stem cells" that mitotically divided to produce a constant supply of precursor cells for formation of the archesporium. Rather, the data suggest that a discrete group of precursor cells was exhausted by their differentiation into archesporial cells and that each of these developed into MMCs that proceeded independently in an asynchronous manner to enter into and progress through meiosis and into megagametophyte development.

The cause of female sterility: Pollinated ears on *mac1* male sterile plants exhibit partial sterility with only about one-fourth or less of the ovules developing into mature kernels. Because the MMCs undergo a normal meiosis, the mutant expression in the ovule appears likely to occur during megaspore differentiation or embryo sac formation. Cytological observations indicate that abnormal development occurs during both of these phases of megagametophyte development. In Figure 6 are shown several tetrads from mutant ovules. Figure 6a shows a

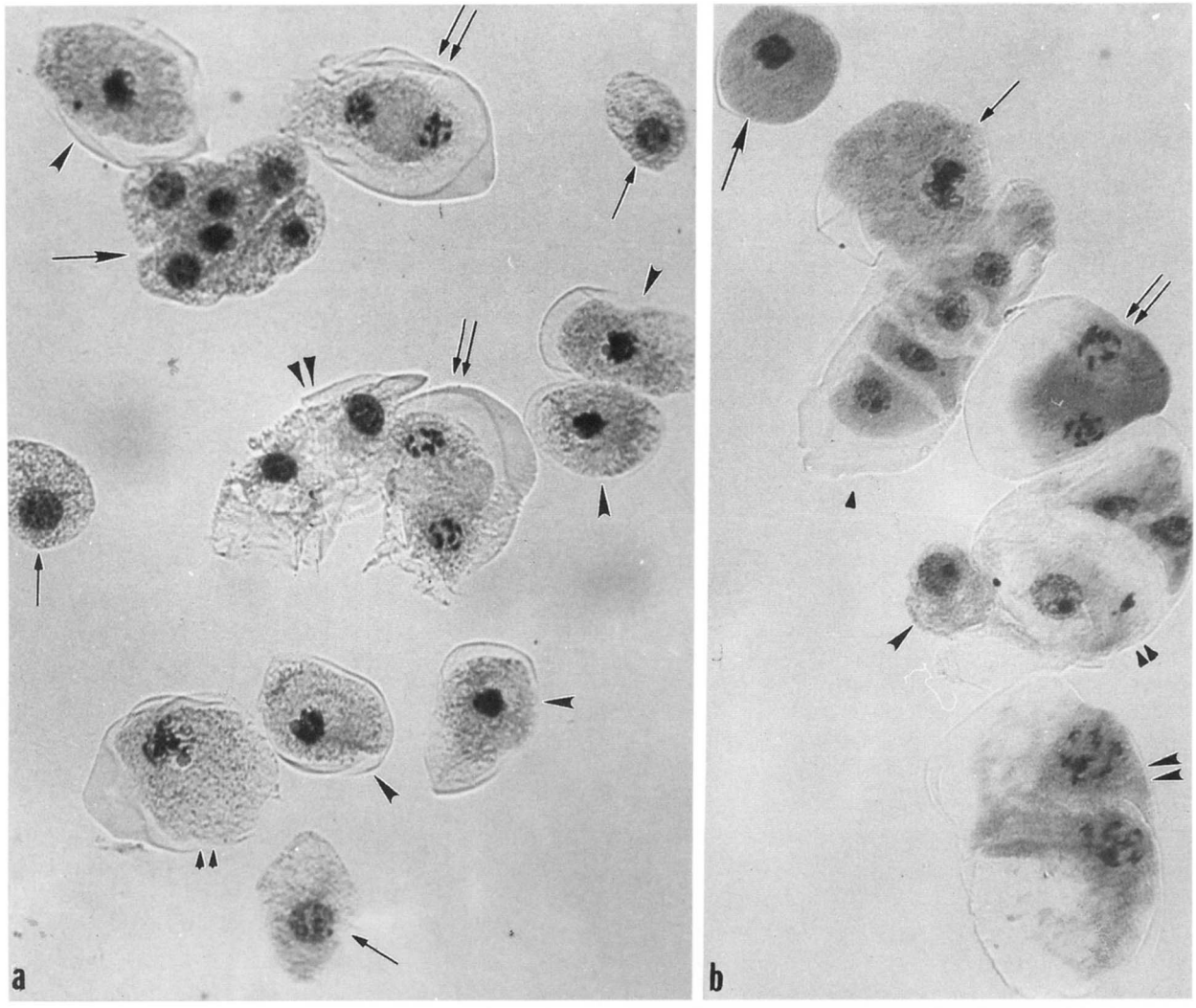

FIGURE 4.—View of sporogenous cells isolated from two mutant ovules with their MMCs at the different stages of meiosis and embryo sac development. (a) Total of 18 archesporial cells and MMCs from the second ovule: a cluster six nucellar cells that appear to be archesporial cells (large arrow), three premiotic MMCs (small arrow), five MMCs at the zygotene stage (arrow head), one MMC at the diplotene stage (double small arrow head), two MMCs at the anaphase I stage (double arrow), and one MMC at the telophase I stage (double large arrow head). (b) A total of seven sporogenous cells from the third ovule: one MMC before meiosis (arrow head), one MMC at the zygotene stage (large arrow), one MMC at the early pachytene (small arrow), one MMC at the late anaphase I (double small arrow), one MMC at the prophase II stage (double arrow head), and two MMCs at the tetrad stage meiosis completed in both products of the one of them (small arrow head), but the other daughter cell after the first meiotic division did not undergo the second meiotic division resulting in the triad cell (double small arrow head).

normal (linear) tetrad configuration of megaspores. However, the tetrad is abnormal because in this tetrad only the upper two of the megaspores have undergone degeneration, whereas in the normal tetrad the upper three megaspores degenerate and only the basal (chalazal-most) megaspore survives, as is shown in Figure 6e. In Figure 6, b–d (bottom tetrad) can be seen abnormal shaped tetrads in which all four megaspores appear to be alive (note their prominent nucleoli). In Figure 6f an abnormal shaped tetrad contains two surviving nuclei and in Figure 6g a four-nucleate embryo sac can be seen to be capped by a surviving megaspore. These observations indicate a malfunctioning in the mechanisms controlling cytokinesis and megaspore survival at the end of megasporogenesis.

A preliminary examination of mature mutant embryo sacs revealed the occurrence of a normal pattern (eight nucleate) embryo sac (Figure 7, a and b, upper embryo sac) as well as degenerating embryo sacs (Figure 7b, lower embryo sac). In addition we observed an abnormally large embryo sac with three times as many nuclei as normally present (Figure 7c).

DISCUSSION

The results of this study lead us to conclude that the *mac1* locus affects the commitment of the hypodermal cells of the ovules to the meiotic pathway and also that this locus affects the development of the female gametophyte. The most significant and interesting feature of the mutational analysis of this gene is its apparent role of acting upon selected hypodermal cells so that they

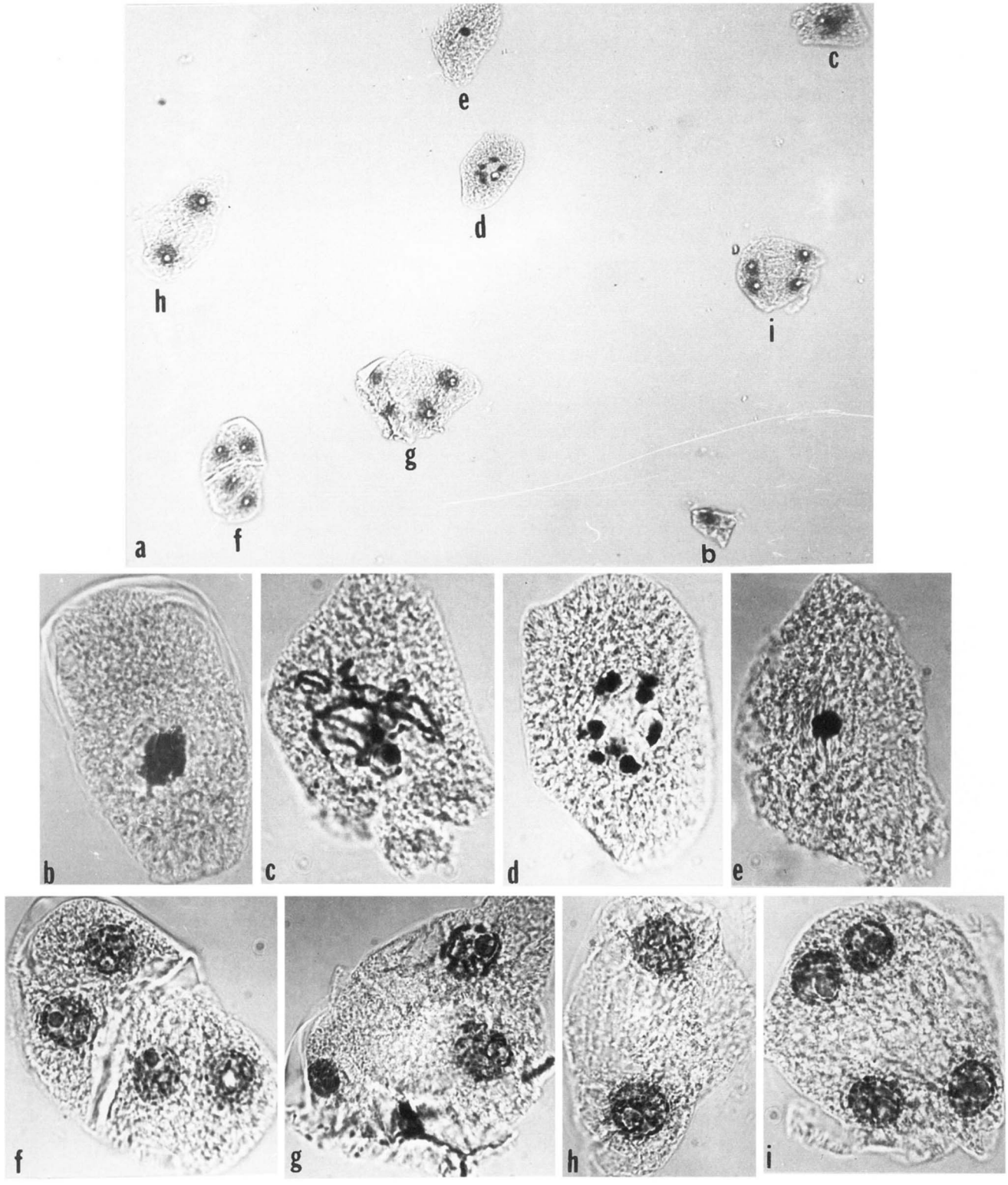

FIGURE 5.—Micrographs of sporogenous cells and cell groups from ovule No. 6 (marked by * in APPENDIX Table A1) of the M6808-30 mutant plant. (a) View of the six MMCs and two embryo sacs at different stages of meiosis and embryo sac development (low magnification). (b–i) Higher magnification of each sporogenous cell or embryo sac shown in (a). (b-g) MMCs at the different stages of meiosis: zygotene (b), pachytene (c), diakinesis (d), metaphase I (e), tetrad stage (f and g). (h-i) Different stages of megagametophyte development: two-nucleate embryo-sac (h), four-nucleate embryo-sac (i).

switch from a sporophytic to a gametophytic destiny. As far as we are aware this is a unique mutation in higher plants. Among the 13 meiotic mutants that we have analyzed cytologically, this is the only mutation affecting the number of archesporial cells formed in the ovule (GOLUBOVSKAYA *et al.* 1992, 1993; I. N. GOLUBOVSKAYA, N. A. AVALKINA, Z. GREBENNIKOVA, and W. F. SHERIDAN, unpublished results).

TABLE 2

Distribution of mutant ovule sporogenous contents between three developmental cell stages: premeiotic, meiosis and embryo sac

Premeiotic	Meiosis I	Meiosis II	Embryo sac	Number of ovules
				0
				13
				16
				5
				1
				11
				14
				2
				1
Total				63

Multicellular higher eucaryotes, including mammals and maize, are faced with a genetic regulatory problem that is not confronted by yeast and other single-celled eucaryotes. Namely, how to genetically switch the developmental path of one or more vegetative (somatic) cells from their mitotic cell cycle to embark up the meiotic developmental pathway. In their review of the meiotic process, RILEY and FLAVELL (1977) noted that "there is little evidence about the cause of the switch from the sequence of somatic cell cycles to meiotic division" and "whatever its nature, the developmental switch from mitotic to meiotic divisions is very effectively protected from error, probably because the conversion involves very many integrated steps. No example has been reported of meiotic divisions being displaced morphologically, spatially, or temporally in eukaryotes" (RILEY and FLAVELL 1977). In animals there is a germ cell lineage, while in plants there is none and cell position appears to be determinative of cell fate. Nevertheless it has been suggested that some "meiosis-inducing substance" is responsible for the induction of meiosis in both animals (BYSKOV 1975; BYSKOV and SAXEN 1986) and in plants (WALTERS 1978, 1985). The nature of such a substance remains unknown. It has been suggested by DICKINSON (1994) that cells seem to acquire a competence to be switched into sporophytic and gametophytic development through dedifferentiation. If such a dedifferentiation process precedes or accompanies the switch to the meiotic pathway and this process involves changes in chromatin and other nuclear and cytoplasmic components, then it might be evidenced by visible changes in the meiotic cell precursors. Consonant with this notion is the widespread occurrence in plants and animals of a gradual increase in the length of the cell cycle, particularly the S phase, in the somatic cells preceding meiosis (BENNETT *et al.* 1971, 1973; BENNETT 1977; RILEY and FLAVELL 1977).

In flowering plants the hypodermal cells of the ovules may be genetically regulated to respond to a meiotic stimulus. In the maize ovule only a single hypodermal cell normally responds to such a hypothetical stimulus (whether it originates externally or arises internally), and this cell proceeds to enlarge both its cytoplasmic and nuclear volume. This enlargement readily identifies that cell that, without further division, will differentiate into the archesporial cell and form the MMC. It is evident, therefore, that in the ovule normally only a single hypodermal cell becomes committed to the meiotic pathway. Our observations that in each mutant ovule several hypodermal cells embark upon the meiotic pathway indicate that the *mac1* locus plays an important role in the commitment of hypodermal cells of the ovule to a meiotic cell destiny. We suggest the *mac1* gene in its normal allelic state controls the response of the hypodermal cells to their cell position and to any meiotic stimulus that might act upon them so as to select or restrict only one of the multiple hypodermal cells of the ovule to a meiotic fate.

The *mac1* gene may act to mediate the generation, transmission or reception of a signal within the developing hypodermal cells of the ovule. In a normal ovule a single hypodermal cell, occupying an apical position at the distal end of the nucellus, may respond to its cell position and either generate its own meiotic commitment signal or become competent to respond to an external meiotic commitment signal. In either case, the *mac1* gene may be activated in this particular hypodermal cell and its expression may result in this cell emitting an inhibitory signal that prevents the other cells of the hypodermal layer from responding to their cell positions and/or a meiotic commitment signal. Because the homozygous *mac1* mutant condition results in the liberation of additional hypodermal cells of the ovule to become committed to meiosis and enter the meiotic pathway, it is most simple to suggest that the normal allele results in the synthesis and diffusion of a component of an inhibitory system. In accord with this hypothesis, the *mac1* mutant allele that we have been studying may be a leaky allele with the timing or strength of expression varying somewhat from one mutant ovule to another resulting in a range of MMCs occurring in mutant ovules. This is consistent with our observations that among the 63 mutant ovules examined the number of MMCs per ovule ranged from three to 21. In those individual mutant ovules with the fewer MMCs, the *mac1* leaky expression might begin earlier in ovule development (or be stronger) and the combined signal emanating from a few committed hypodermal cells would suffice to inhibit additional hypodermal cells from becoming committed to developing into archesporial cells. On the other hand, in those individual mutant ovules with the higher number of MMCs, the onset of *mac1* leaky expression might be later in ovule development (or be weaker) and only after several or more of the hypodermal cells had become committed to the meiotic pathway would their combined signal be strong enough to inhibit the commitment of additional hypodermal cells to the meiotic pathway. If the *mac1* mutant allele is leaky, then the above hypothesis might

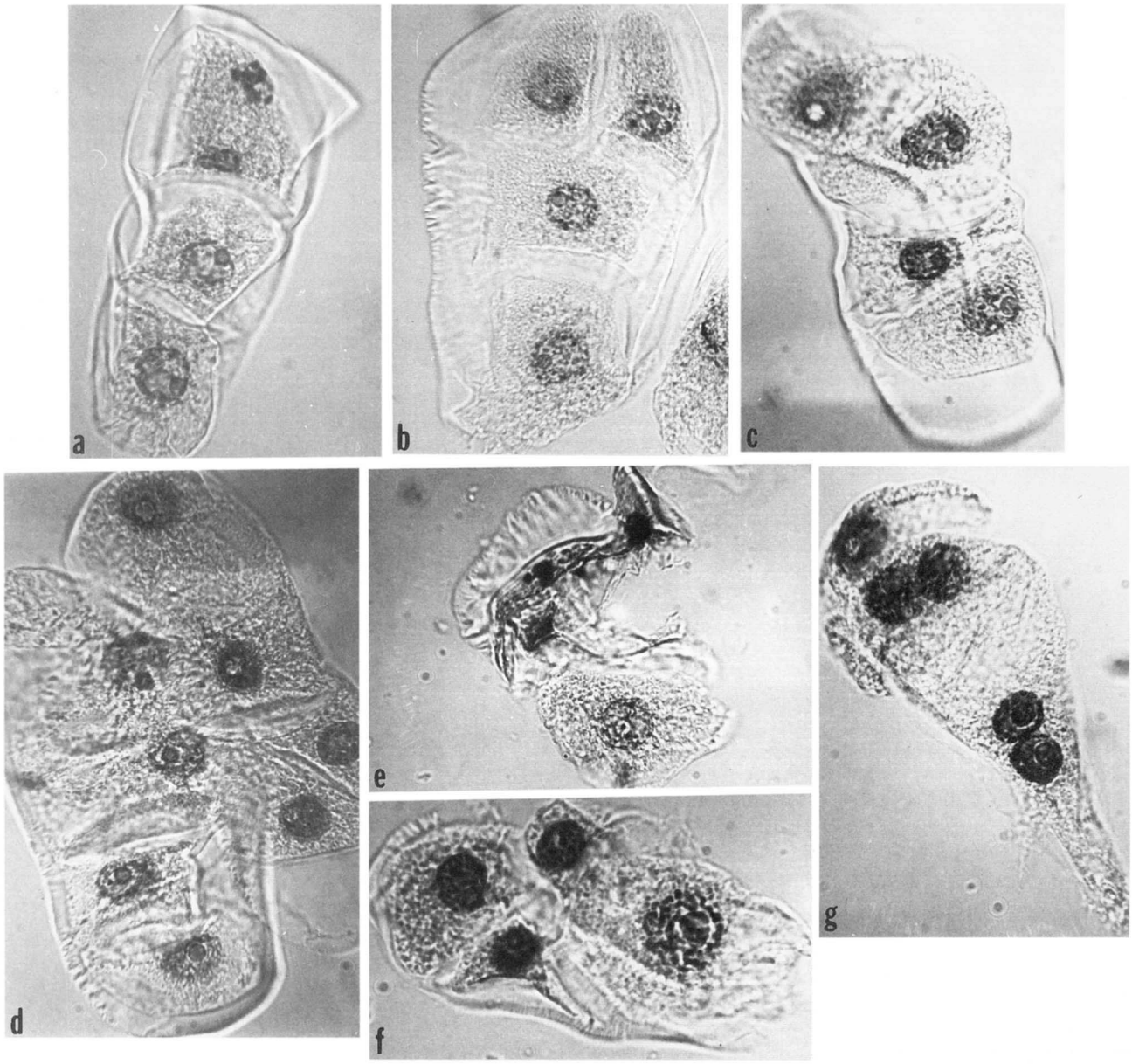

FIGURE 6.—Different shapes of megaspore tetrads in the *mac1* mutant. (a) Linear shaped of tetrad. (b) T-shaped tetrad. (c) Intermediate shaped tetrad as a result of a nontransverse second cytokinesis. (d) Linear and intermediate shaped tetrads. In this tetrad as in a–c, all four megaspores are still alive and do not show any evidence of degeneration. (e) Tetrad of megaspores, only the bottom megaspore is still alive, the sister megaspores have all degenerated. (f) Abnormal shaped tetrad, with the two end megaspores still alive. (g) Four-nucleate embryo sac, but with a surviving sister megaspore present as a cap above the embryo-sac.

receive support from analysis of the effect of differing dosage of the mutant allele on the number of MMCs per mutant ovule. The number would be expected to be greater in mutant plants *hypoploid* for chromosome arm *10S* as compared to the number of MMCs per ovule in mutant diploid plants. But in mutant *hyperploid* plants the number of MMCs per ovule would be expected to be less than that observed in mutant diploid plants. We are pursuing these analyses.

An alternative to the inhibitory signal hypothesis is that in mutant ovules multiple hypodermal cells misinterpret their cell position so that not only the apical-most hypodermal cell but adjacent cells as well may generate their own meiotic commitment signal or become competent to respond to an external meiotic commitment signal. According to this line of thinking, the normal *mac1* allele would play a role in the recognition by a hypodermal cell of its cell position and its appropriate response. The range in number of hypodermal cells becoming committed to the meiotic pathway could be a result of the variable degree of leakiness in the expression of the mutant allele among different mutant ovules.

A second feature of mutant ovules warrants brief discussion. Whereas the meiocytes of normal anthers proceed through prophase I of meiosis with a high degree of synchrony, the multiple meiocytes (MMCs) of mutant ovules proceed through a normal meiotic prophase I but

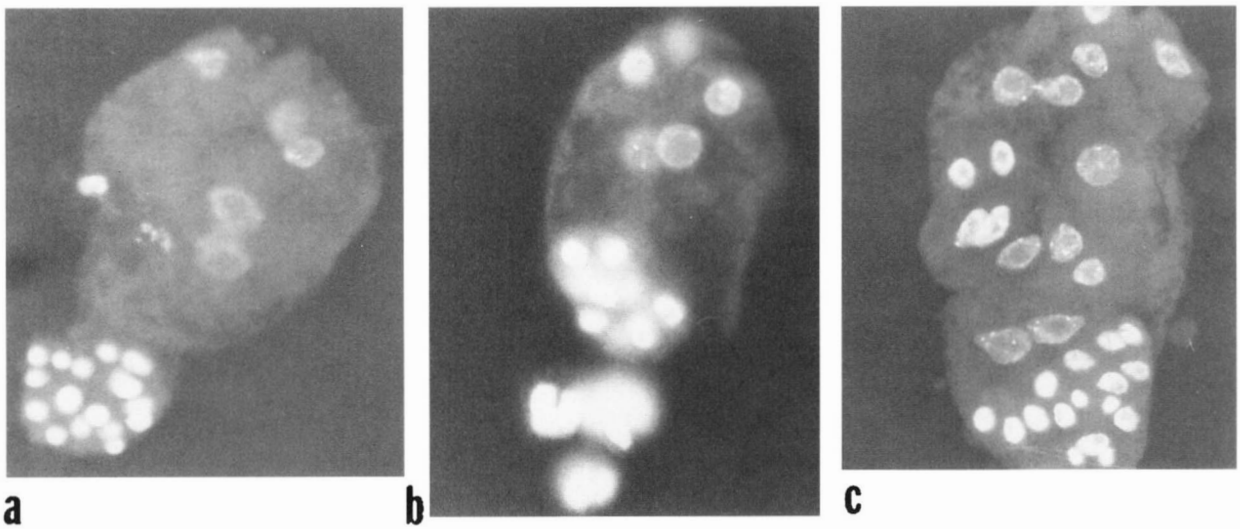

FIGURE 7.—Isolated normal and mutant mature embryo sacs with DAPI staining of nuclei. (a) Normal mature embryo sac containing two synergids, an egg-cell, the two-nucleate central cell, and the antipodal complex of cells (bright cluster of nuclei at the bottom). Note the presence of a bright staining contaminant nucellar cell nucleus at the left edge of the central cell. (b) Two embryo sacs are seen: one (upper) is normally developed with two synergids, one egg-cell, a two-nucleate central cell and an antipodial complex; the other embryo sac (bottom) is degenerated and contains dense brightly fluorescent nuclei. (c) An abnormal multicellular mature embryo sac that likely resulted from the development of three megaspores into this single embryo sac (chimeric development). Note that, excluding the antipodal complex (bottom group of brightly staining nuclei), there are 15 nuclei in this embryo sac. Compare with the corresponding five nuclei (excluding the antipodal complex) shown in the normal embryo sac in a.

they do so asynchronously. Whether this is a direct result of the *mac1* mutation or a reflection of possible structural isolation of the MMCs in mutant ovules so that they proceed into meiosis independent of each other remains to be determined. Future ultrastructural analyses of mutant and normal ovules may clarify whether the multiple MMCs of mutant ovules lack the cytoplasmic connections that normally connect prophase I stage male meiocytes in angiosperms (HESLOP-HARRISON 1964) and that are thought to function in the maintenance of meiotic synchrony (HESLOP-HARRISON 1966). In the individual ovules of *P. californica*, WALTERS (1962) observed that the multiple megasporocytes that entered into meiosis proceeded through it in an asynchronous manner.

An additional observation that merits comment is the female partial sterility of *mac1* mutant plants. The failure in kernel development that we have observed on mutant ears despite the abundant pollination of their silks appears to result from a high frequency in failure of development of functional ovules. This failure is a sporophytic trait (as is the male sterility) inasmuch as it is expressed only in the homozygous mutant plants. Our preliminary observations indicate that abnormal megaspore alignment and fate as well as abnormal embryo sac formation occur in mutant ovules. The observation of a mutant embryo sac with three times the normal number of nuclei indicates that three (or more) megaspores may participate in the formation of a single embryo sac and that each of the three megaspore nuclei may undergo three divisions. Future examination of these processes should reveal their frequency and provide greater details of the abnormalities. Because the mutant microsporocytes fail to produce microspores we cannot draw any conclusions at this time as to the possible role of the sporophytic expression of the *mac1* locus in male gametophyte development. However, the occurrence of the partial sterility on pollinated ears as well as our preliminary cytological studies on mature mutant ovules indicate that not only does the *mac1* locus play an important role in the commitment of somatic cells to the meiotic pathway but that normal *mac1* gene expression before or during meiosis (in sporophytic cells) is important for the subsequent normal female gametophyte development and formation of functional embryo sacs. The mutant phenotypes of multiple megasporocytes and embryo sacs per ovule and a large number of free nuclei per embryo sac are primitive traits (EAMES 1961). The *mac1* gene may prove to be of interest in understanding the divergence of the angiosperms from their related higher vascular plants. Finally, we would note that the effect of the *mac1* mutation on anther development is being studied so as to obtain insight as to the cause of failure of microsporocyte development. At this time we suggest that the differences in the female and male mutant phenotypes likely reflect the differences in hypodermal cell behavior and in the timing of commitment to meiosis in maize ovules and anthers. This mutation is putatively tagged with a Mutator element and we have initiated an effort to clone this gene.

We thank DON AUGER and GUY FARISH for their assistance in the seed room and the experimental field. We thank BING-QUAN HUANG for the photographs of the plastic sectioned ovules and of the DAPI-stained embryo sacs. We are grateful to the National Science Foundation (NSF) Office of International Programs and the NSF Developmental Biology Program for grant No. INT-9016633 supporting the U.S.-Russian Workshop on Maize Development that facilitated our collaboration. This research was supported in part by U.S. Depart-

ment of Agriculture Grant 94-37304-1045 to W.F.S. and by International Science Foundation Grant NXU000 to I.N.G.

LITERATURE CITED

ANDERSON, E. G., 1938 Translocations in maize involving chromosome 9. Genetics **23:** 307–313.

ANDERSON, E. G., 1956 The application of chromosomal techniques to maize improvement. Brookhaven Symp. Biol. **9:** 23–36.

BECKETT, J. B., 1994 Locating recessive genes to chromosome arm with B-A translocations, pp. 315–327 in *The Maize Handbook,* edited by M. FREELING and V. WALBOT. Springer-Verlag, New York.

BEDINGER P., and S. D. RUSSELL, 1994 Gametogenesis in maize, pp. 48–61 in *The Maize Handbook,* edited by M. FREELING and V. WALBOT. Springer-Verlag, New York.

BENNETT, M. D., 1977 The time and duration of meiosis. Phil. Trans. Roy. Soc. Lond. B **277:** 201–226.

BENNETT, M. D., V. CHAPMAN and R. RILEY, 1971 The duration of meiosis in pollen mother cells of wheat, rye, and Triticale. Proc. Roy. Soc. Lond. B **178:** 259–275.

BENNETT, M. D., R. A. FINCH, J. B. SMITH and M. K. RAO, 1973 The time and duration of female meiosis in wheat, rye, and barley. Proc. Roy. Soc. Lond. B **183:** 301–319.

BYSKOV, A. G., 1975 The role of the rete ovarii in meiosis and follicle formation in the cat, mink, and ferret. J. Reprod. Fertil. **45:** 201–209.

BYSKOV, A. G., and L. SAXEN. 1986 Induction of meiosis in fetal mouse testes in vitro. Dev. Biol. **52:** 193–200.

COOPER, D. C. 1937 Macrosporogenesis and embryo sac development in *Euchlaena mexicana* and *Zea mays.* J. Agric. Res. **55:** 539–551.

COULTER, J. M., and C. J. CHAMBERLAIN, 1903 *Morphology of Angiosperms.* Appleton, New York.

DAVIS, G. L., 1966 *Systematic Embryology of the Angiosperms.* Wiley, New York.

DICKINSON, H. G. 1994 The regulation of alternation of generation in flowering plants. Biol. Rev. **69:** 419–442.

DUMAS, C., and H. L. MOGENSEN, 1993 Gametes and fertilization: maize as a model system for experimental embryogenesis in flowering plants. Plant Cell **5:** 1337–1348.

EAMES, A. J., 1961 *Morphology of the Angiosperms.* McGraw-Hill, New York.

GIFFORD, E. M., and A. S. FOSTER, 1987 *Morphology and Evolution of Vascular Plants.* W. H. Freeman, New York.

GOLUBOVSKAYA, I. N., N. AVALINKA and W. F. SHERIDAN, 1992 Effects of several meiotic mutants on female meiosis in maize. Dev. Genet. **13:** 411–424.

GOLUBOVSKAYA, I., Z. K. GREBENNIKOVA, N. A. AVALKINA and W. F. SHERIDAN, 1993 The role of the *ameiotic1* gene in the initiation of meiosis and in subsequent meiotic events in maize. Genetics **135:** 1151–1166.

HESLOP-HARRISON, J., 1964 Cell walls, cell membranes, and protoplasmic connections during meiosis and pollen development, pp. 39–47 in *Pollen Physiology and Fertilization,* edited by H. F. LINSKENS. North Holland Publishing Co., Amsterdam.

HESLOP-HARRISON, J., 1966 Cytoplasmic continuities during spore formation in flowering plants. Endeavour **25:** 65–72.

HOLM, P. B., S. KNUDSEN, P. MOURITZEN, D. NEGRI, F. L. OLSEN *et al.,* 1994 Regeneration of fertile barley plants from mechanically isolated protoplasts of the fertilized egg. Plant Cell **6:** 531–543.

HOLM, P. B., S. KNUDSEN, P. MOURITZEN, D. NEGRI, F. L. OLSEN *et al.,* 1995 Regeneration of the barley zygote in ovule culture. Sex. Plant. Reprod. **8:** 49–59.

HUANG, B.-Q., and S. D. RUSSELL, 1989 Isolation of fixed and viable eggs, central cells and embryo sacs from ovules of *Plumbago zeylanica.* Plant Physiol. **90:** 9–12.

HUANG, B.-Q., and S. D. RUSSELL, 1992 Female germ unit: organization, isolation and function. Int. Rev. Cytol. **140:** 133–193.

HUANG, B.-Q., and S. D. RUSSELL, 1993 Polarity of nuclear and plastid DNA in megasporogenesis and megagametogenesis of *Plumbago zeylanica.* Sex. Plant Reprod. **6:** 205–211.

HUANG, B.-Q., and W. F. SHERIDAN, 1994 Female gametophyte development in maize: microtubular organization and embryo sac polarity. Plant Cell **6:** 845–861.

IMMER, F. R. 1930 Formulae and tables for calculating linkage intensities. Genetics **15:** 81–98.

KIESSELBACH, T. A., 1949 The structure and reproduction of corn. Univ. Nebraska Coll. Agric. Exp. Station Res. Bull. **161:** 1–96.

KRANZ, E., and H. LORZ, 1993 In vitro fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. Plant Cell **5:** 739–746.

MAHESHWARI, P., 1950 *An Introduction to the Embryology of the Angiosperms.* McGraw-Hill, New York.

RANDOLPH, L. F., 1936 Developmental morphology of the caryopsis of maize. J. Agr. Res. **53:** 881–916.

REDEI, G. P., 1982 *Genetics.* MacMillan Publishing Co., New York.

REISER, L., and R. L. FISCHER, 1933 The ovule and embryo sac. Plant Cell **5:** 1291–1301.

RILEY, R., and R. B. FLAVELL, 1977 A first view of the meiotic process. Phil. Trans. Roy. Soc. Lond. B. **277:** 191–199.

RUSSELL, S. D. 1993 The egg cell: development and role in fertilization and early embryogenesis. Plant Cell **5:** 1349–1359.

WALTERS, J. L., 1962 Megasporogenesis and gametophyte selection in *Paeonia californica.* Amer. Jour. Bot. **49:** 787–794.

WALTERS, M. S., 1978 Meiosis readiness in *Lilium longiflorum* "Croft". Chromosoma **67:** 365–391.

WALTERS, M. S., 1985 Meiosis readiness in *Lilium.* Can. J. Genet. Cytol. **27:** 33–38.

WEST, M. A. L., and J. J. HARADA, 1993 Embryogenesis in higher plants: an overview. Plant Cell **5:** 1361–1369.

Communicating editor: J. A. BIRCHLER

APPENDIX TABLE A1

Number of stage distribution of MMCs and embryo sacs (ESs) in normal and mutant ovules

Plant no. and ovule no.											Embryo sac stage / No. of nuclei			
Mutant	Premeiosis	L	Z	P	Dip	Dia	MI	A-TI	Dyad	Tetrad	2	4	8	Total
6808-30														
1	1	–	–	–	–	–	–	–	–	3	–	–	–	4
2	–	–	–	1	–	–	–	–	1	3	–	–	–	5
3	1	10	–	–	–	–	–	–	–	5	–	–	–	16
4	–	–	2	–	1	–	–	–	–	3	–	–	–	6
5	–	–	2	–	–	–	–	–	–	2	–	3	–	7
6*	–	–	1	1	–	1	1	–	–	2	5	2	–	13
7	–	–	–	–	–	–	–	–	–	1	3	–	2	6
8	–	–	–	–	–	–	–	–	–	4	1	1	–	6
9	–	–	1	–	–	–	–	–	–	4	1	2	–	8
10	–	–	6	–	–	1	–	–	–	–	2	2	1	12
11	–	–	2	4	–	–	–	1	–	2	–	–	–	9
12	–	–	–	1	–	1	–	–	–	2	1	4	9	9
13	–	–	–	–	–	–	2	–	–	1	3	1	1	6
14	–	–	3	1	–	–	–	–	–	4	1	2	–	11

APPENDIX TABLE A1

Continued

Plant no. and ovule no.											Embryo sac stage			
											No. of nuclei			
Mutant	Premeiosis	L	Z	P	Dip	Dia	MI	A-TI	Dyad	Tetrad	2	4	8	Total
6808-30														
15	–	–	1	–	–	–	–	– –	– –	– – –	3	2	–	6
16	–	–	4	2	–	–	1	– –	– –	1	–	–	–	8
17	–	–	2	–	–	–	–	– –	1	– –	– – –	–	–	3
18	–	–	2	4	–	1	–	– –	2	1	– – –	–	–	10
19	–	–	6	1	–	–	–	1	– –	1	–	–	–	9
20	–	–	2	1	–	–	–	– –	– –	2	–	–	–	5
21	–	–	–	3	–	–	1	– –	– –	5	1	1	–	11
22	12	–	6	1	–	–	–	– –	– –	– – –	–	–	–	19
23	1	–	–	–	–	1	–	– –	– –	4	2	3	1	12
24	1	–	1	2	–	1	–	– –	– –	– – –	1	1	1	8
25	–	–	–	–	–	–	1	– –	– –	1	2	4	–	8
26	2	–	2	1	–	–	1	– –	– –	7	1	–	–	14
Total	18	10	43	22	1	6	5	2	4	58	27	28	6	230
M6734-6														
1	5	–	2	2	–	–	1	– –	– –	– –	– – –	–	–	10
2	11	–	1	1	–	–	–	1	– –	– –	– – –	–	–	14
3	4	–	2	1	–	1	–	– –	– –	– –	– – –	–	–	8
4	5	–	3	–	–	1	–	– –	– –	– –	– – –	–	–	9
5	6	–	4	–	1	–	–	– –	– –	– –	– – –	–	–	11
6	–	–	–	–	1	–	–	1	2	– –	– – –	–	–	4
7	7	–	–	–	–	1	–	– –	1	2	– – –	–	–	11
8	1	–	3	–	1	–	1	– –	–	1	– – –	–	–	7
9	10	–	1	–	–	–	–	– –	1	1	– – –	–	–	13
10	6	–	–	3	–	–	–	– –	– –	– –	– – –	–	–	9
11	–	–	2	–	–	1	–	– –	1	1	– – –	–	–	5
12	2	–	3	1	–	–	–	– –	– –	2	– – –	–	–	8
13	5	–	4	–	–	–	–	– –	– –	2	– – –	–	–	11
14	–	–	7	1	1	1	–	– –	– –	1	– – –	–	–	11
15	–	–	4	2	–	–	–	– –	– –	2	2	–	–	10
16	17	–	3	1	–	–	–	– –	– –	– –	– – –	–	–	21
17	–	–	1	2	1	1	–	– –	– –	1	– – –	1	1	8
18	2	–	2	–	–	–	–	– –	– –	– –	– – –	–	–	4
19	2	–	2	–	–	–	–	– –	– –	4	– – –	–	–	8
20	9	–	6	1	–	–	–	– –	2	– –	– – –	–	–	18
21	1	–	2	1	1	–	–	– –	1	– –	– – –	–	–	6
22	2	–	2	3	–	–	–	1	– –	– –	– – –	–	–	8
23	1	–	3	1	–	–	–	1	1	2	– – –	–	–	9
24	4	–	2	1	–	–	1	– –	– –	1	– – –	–	–	9
25	1	–	2	2	1	–	–	1	– –	3	– – –	–	–	10
26	2	–	3	–	–	2	–	– –	– –	3	– – –	–	–	10
27	8	–	1	–	1	2	–	– –	– –	– –	– – –	–	–	12
28	5	–	5	1	–	–	–	– –	1	3	– – –	–	–	15
29	6	–	4	2	–	–	–	– –	– –	2	– – –	–	–	14
30	2	–	5	–	–	1	–	– –	– –	1	– – –	–	–	9
Total	124	–	79	27	8	11	3	5	10	32	2	1	1	303
M6808-6														
1	–	–	2	–	–	–	1	– –	1	3	3	–	–	8
2	–	–	2	1	–	1	–	1	– – –	–	1	1	–	7
3	–	–	–	–	–	–	–	– –	– –	– –	1	5	–	6
4	–	–	–	1	–	–	–	– –	– –	1	3	–	–	5
5	–	–	–	1	–	–	–	– –	– –	– –	– – –	1	2	4
6	–	–	3	–	–	–	–	– –	– –	3	– – –	–	–	6
7	–	–	2	2	–	–	–	2	1	3	1	–	–	11
Total	–	–	9	5	–	1	1	3	2	10	7	7	2	47
TOTAL	142	10	131	54	9	18	9	10	16	100	36	36	9	580
Normal										MII-TII				
M6808-2			5	1	–	1	–	–	2	2 5	1	3	–	20
–4			2	2	–	–	2	1	1	–10	1	–	2	21
–5			2	3	–	–	–	1	–	1 2	4	1	1	15
–31			3	1	–	2	–	–	2	–2	3	1	–	14
TOTAL			12	7	0	3	2	2	5	3 19	9	5	3	70

Abbreviations of meiotic stages: L, leptotene; P, pachytene; Dia, diakinesis; MII-TII, metaphase II-telophase II; Z, zygotene; Dip, diplotene; MI, metaphase I; A-TI, anaphase-telophase I.