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# Endosperm Development after Fusion of Isolated, Single Maize Sperm and Central Cells in Vitro

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**We demonstrate here the possibility of endosperm development in vitro after the fusion of pairs of an isolated sperm and an isolated central cell of maize. The occurrence of karyogamy and the time course of the fusion of sperm and central cell nuclei are presented. The fusion of the sperm nucleus occurred either with one of the two polar nuclei or with the secondary nucleus and was completed within 2 hr after in vitro cell fusion. The in vitro study of early events after cell and nuclear fusion indicates that the resulting primary endosperm cell develops into a characteristic tissue capable of self-organization apart from the mother tissue. The technology presented here opens the way for new cellular and molecular studies, especially of early events after sperm and central cell fusion. These studies should lead to a better understanding of the processes of double fertilization and endosperm development.**

## INTRODUCTION

Endosperm development is of great interest and importance both in agriculture, for biotechnical improvement, and in developmental biology research (e.g., Kowles and Phillips, 1988; Olsen et al., 1992; Lopes and Larkins, 1993; Clore et al., 1996). It has been extensively studied morphologically, ultrastructurally, and histochemically; however, there is a lack of information on sperm–central cell fusion and on early molecular events after fertilization. This is due to limited experimental access to the central cell, which is located in the embryo sac deeply embedded in nucellar tissue.

In angiosperms, the two sperm cells of a pollen grain or tube are involved in fertilization: one fuses with the egg and the other with the central cell (reviewed in Russell, 1992). These processes, well known as double fertilization, result in the formation of an embryo and endosperm tissue (e.g., Goldberg et al., 1994). Double fertilization in maize after pollination in the plant has been widely investigated both cytologically and ultrastructurally (e.g., Rhoades, 1934; Diboll and Larson, 1966; Diboll, 1968; Van Lammeren, 1986; Van Lammeren and Kieft, 1987; Mól et al., 1994) and during in vitro ovary culture (Schel and Kieft, 1986).

Maize endosperm development can be divided into four stages, as described, for example, by Clore et al. (1996). During stage I (after central cell fertilization until 3 days after pollination [DAP]), rapid nuclear divisions without cell wall formation occur (syncytium formation). Cell wall formation around the single nuclei takes place, resulting in a tissue with uninucleate cells during stage II (cellularization, 3 to 5 DAP), followed by stage III, which is characterized by the occurrence of mitotic divisions until ~12 DAP in the centrally located cells and until 20 to 25 DAP in the peripheral tissue. Starch grains and protein bodies accumulate in the center of the tissue. This process starts during stage III at ~10 DAP and continues during stage IV, when the maize kernels desiccate and cell death of the endosperm occurs.

With experimental access to isolated gametes under more controlled conditions, investigations of early events, which are timed precisely after gamete fusion, are now possible. In maize, zygotes produced in vitro divide (Kranz et al., 1991a; Kranz and Lörz, 1994; Digonnet et al., 1997) and can regenerate via direct, primary embryogenesis into fertile plants (Kranz and Lörz, 1993). Also, isolated maize central cells occasionally were isolated in earlier experiments. However, because of the limited amount of these cells, central cells were fertilized in vitro only in a few cases (Kranz et al., 1991b). Therefore, systematic biochemical analyses with this material have not been possible until now, and the development of endosperm after in vitro fusion has not been documented in angiosperm species. Detailed studies on sperm–central cell fusion can now be performed. Also, endosperm formation in vitro makes it possible to study very early events after the fusion of the sperm with the central cell under defined conditions. Moreover, such events can now be studied independently of any mother tissue and of the zygote.

In our study with maize, we present (1) the development of a procedure for the reproducible and efficient isolation of

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central cells; (2) the technology for handling, selection, and individual fusion of sperm and central cells; (3) the occurrence and the time course of karyogamy in the central cell; (4) the development of in vitro–produced endosperm in individual culture; (5) the occurrence of triploid tissue; and (6) morphological and cytological characterization of in vitro–produced endosperm.

Based on the results of endosperm formation reported in this study, it is now possible to perform the two fertilization processes in vitro that were discovered in higher plants by Nawaschin (1898) and Guignard (1899).

## RESULTS

### Isolation and Characterization of the Central Cell

Maize central cells were reproducibly isolated from ovular tissue at various developmental stages. Ovules were 2 to 3 mm in diameter, and silk emergence lengths were 9 to 18 cm. Compared with egg cell isolation, some modifications were required for the efficient isolation of maize central cells. Whereas the treatment of nucellar tissue with cell wall–degrading enzymes for efficient egg cell isolation was not >30 min, a longer duration for this treatment (45 min) was optimal for central cell isolation. In addition, it was advantageous to use a cell wall–degrading enzyme mixture with an osmolarity higher than that used for egg cell isolation. Plasmolysis resulted in the separation of the central cell membrane from the embryo sac wall, which enabled us to improve the manual isolation step.

In contrast to egg cell isolation, only the nucellar cells at the micropylar end of the embryo sac were removed from the tissue pieces, as demonstrated in Figure 1A at left. Starting at the chalazal end near the antipodal cells, the central cell was then pushed by a microneedle toward the micropylar end of the embryo sac, where it was liberated and became spherical (Figures 1B at left, 2A, and 2B). This manual procedure was necessary and determined the final yield of isolated central cells, because the hard embryo sac wall was not digested after treatment with the enzyme mixture. This wall showed green fluorescence after aniline blue staining, indicating that callose is a component of wall material. Routinely, three to eight central cells were isolated from ~160 tissue pieces within 2 to 3 hr. A total of 355 central cells were isolated in this study.

The isolated central cell had no cell wall and became spherical after isolation. Thus, it is a protoplast. Compared with other cells of the embryo sac and with pollen grains, sperm cells, and somatic cells of a maize plant, the central cell is very large, as demonstrated in Table 1 and Figures 2C to 2E. Because of the large size of the maize central cell protoplast, careful handling was necessary, especially during cell transfer. The cell was highly vacuolated. Nuclei in the isolated central cell were observed only occasionally (Figure

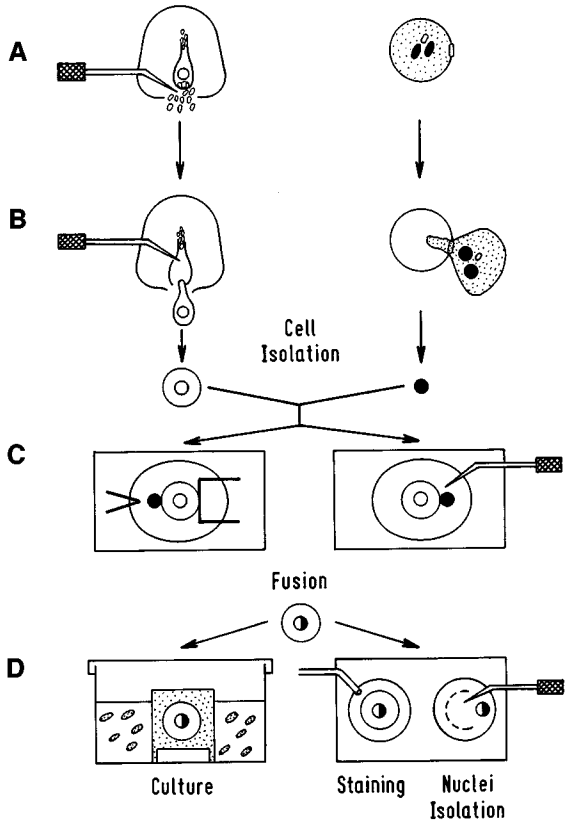


**Figure 1.** General Procedures for the Isolation and Fusion of Sperm and Central Cells, Nuclei Isolation and Staining, and Culture of in Vitro–Fertilized Central Cells in Maize.

**(A)** The central cell is manually isolated after dissection of the ovular tissue piece and treatment with cell wall–degrading enzymes. Nucellar cells have been removed mainly at the micropylar pole of the embryo sac, as shown at left. Sperm cells are isolated from the mature pollen grain by osmotic shock in mannitol, as shown at right.

**(B)** The central cell is pushed out of the embryo sac during the final manual step, as shown at left. Sperm cells are selected after bursting, as shown at right.

**(C)** Fusion of a sperm with a central cell is performed on coverslips either in a microdroplet of a mannitol solution with electrical pulses using microelectrodes, as shown at left, or in a microdroplet of a calcium-containing mannitol solution after manual alignment of the cells by using a microneedle, as shown at right.

**(D)** The in vitro–fertilized central cell is cultured in a Millicell dish that was previously inserted in a larger dish containing feeder cells, as shown at left. The central cell is stained by the addition of a fluorochrome-containing solution by use of a microcapillary, and nuclei are manually isolated by using a microneedle in microdroplets on a coverslip, as shown at right.

2F) because they were surrounded by the main cytoplasm, including the other cell organelles. This cluster, which is rich in starch grains, was located mainly at the cell periphery. Thus, after isolation, the central cell still maintained its polarity.

The organelle cluster was stained with fluorochromes; this treatment masked the nuclei, thus making it difficult to discriminate between fluorescent signals of the nuclei and the other organelles. Therefore, the isolation of nuclei and the removal of other cell organelles proved to be a useful procedure to enable an easy and clear examination of nuclei of the unfertilized central cells, as demonstrated in Figure 1D at right. In three independent experiments, 22 nuclei from

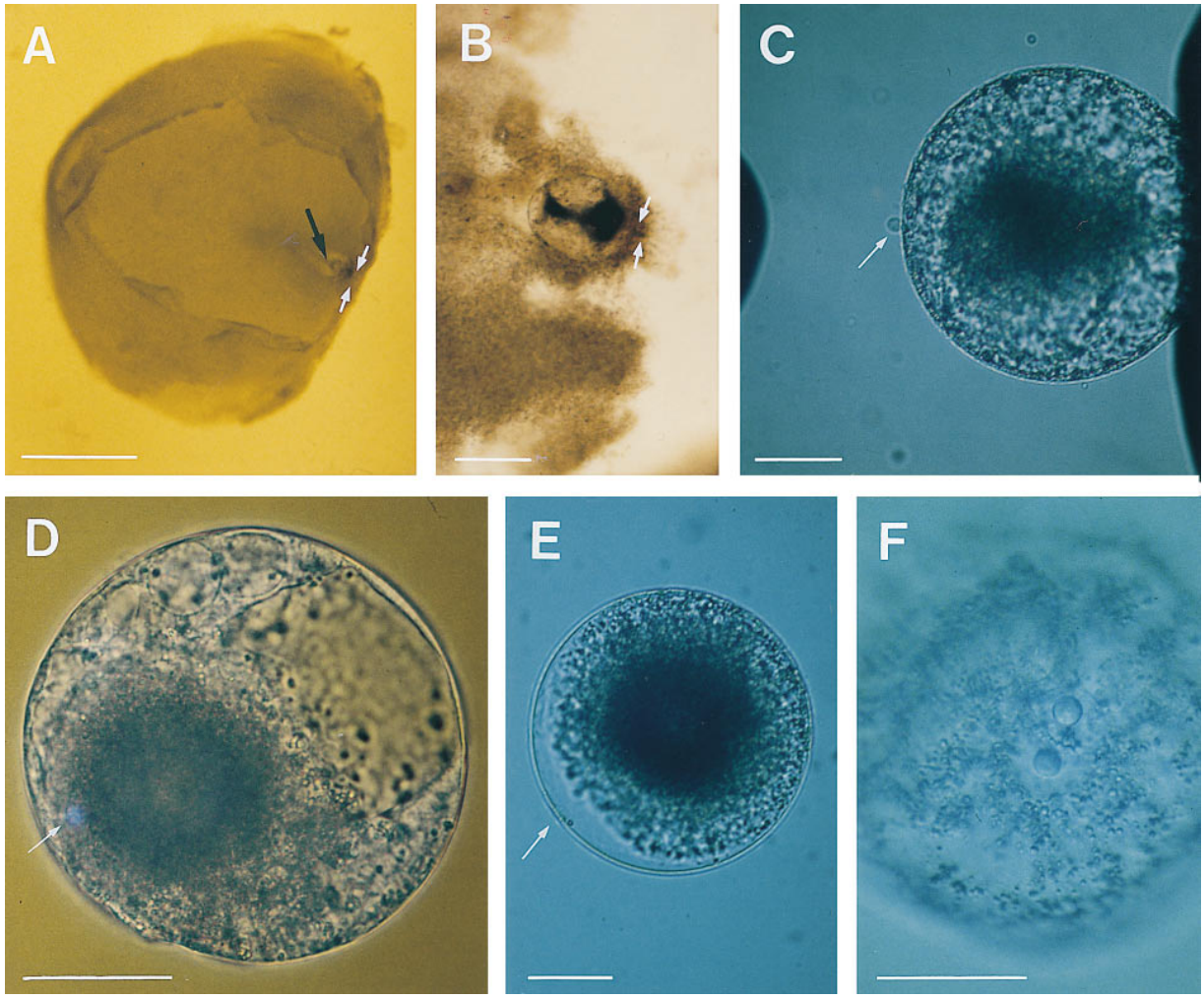


**Figure 2.** Isolation of Central Cells and in Vitro Cell Fusion of Central Cells with Sperm Cells.

Central cell donor line A188 and sperm donor line Pirat were used.

**(A)** Ovular tissue piece containing the embryo sac after incubation in an enzyme mixture of cell wall–degrading enzymes. Nucellar cells near the embryo sac in the micropylar region have been removed (white arrows). In the following step, the central cell is pushed toward the micropylar pole with a microneedle, starting near the chalazal end of the embryo sac (black arrow). Bar = 1 mm.

**(B)** Manual isolation of the central cell. The nucellar cells around the embryo sac, especially near the micropylar area, as well as the egg apparatus have been removed with a microneedle (white arrows). Bar = 200 μm.

**(C)** Electrical alignment of a sperm and a central cell before fusion. The arrow indicates the sperm cell. Bar = 50 μm.

**(D)** Sperm inside the central cell. The location of the sperm nucleus is indicated by an arrow. One hour after sperm–central cell fusion, the cell was stained with DAPI, followed by light and epifluorescence microscopic analysis. Bar = 50 μm.

**(E)** Central cell after fusion with a sperm cell in a calcium (5 mM $CaCl_2$)-containing mannitol solution. The arrow indicates the peripheral location of the sperm cell inside of the central cell. Bar = 50 μm.

**(F)** Two polar nuclei, positioned close to each other, at the periphery of a 1-hr-old fusion product. Bar = 50 μm.

**Table 1.** Cell Size and Volume of Isolated Egg, Sperm, and Central Cells of Maize

| Cell[a] | No. | Diameter[b] $\bar{x} \pm \bar{x}\ \sigma n - 1$ μm | Volume[b] $\bar{x} \pm \bar{x}\ \sigma n - 1$ μm³ | pL | Ratio |
|---|---|---|---|---|---|
| Central cell[c] | 20 | 203.7 ± 13.9 | 4,480,955 ± 816,593 | 4481 | Central cell volume/egg cell volume 27 |
| Egg cell[d] | 15 | 67.9 ± 2.6 | 164,461 ± 16,943 | 164 | Egg cell volume/sperm cell volume 744 |
| Sperm cell | 10 | 7.5 ± 0.08 | 221 ± 7.2 | 0.2 | Central cell volume/sperm cell volume 20,276 |

[a] Line A188.

[b] $\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}; \sigma_{n-1} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}$.

[c] Isolated from ovules 2.0 to 3.0 mm in diameter.

[d] Isolated from ovules 2.5 to 3.5 mm in diameter.

isolated central cells of unpollinated ovules (ovule size of 2.2 to 3 mm; emerged silk length of 12 to 14 cm) were isolated and stained with 4′,6-diamidino-2-phenylindole (DAPI). Two polar nuclei, positioned close to each other, were observed in 18 central cells (82%; Figures 3A and 3B), and a secondary nucleus was seen in four unfertilized central cells (18%; Figure 3C). After the isolation of the unfertilized central cell nuclei, the diameter of a polar nucleus was 15 to 20 μm, and that of a secondary nucleus was 29 μm. The polar and secondary nuclei had one nucleolus. The diameter of the nucleolus of a polar nucleus was 9 to 13 μm, and that of the secondary nucleus was 15 to 17 μm with a large pore of 8 μm. The volume of the secondary nucleus was 12,775 μm³. The volume of its large nucleolus was 2146 μm³. The diameter of the nuclei of the in vitro zygotes that were isolated 1 hr after the egg–sperm in vitro fusion was 13 to 14 μm, and that of the nucleoli was 7 μm.

### Fusion of Sperm and Central Cell

Sperm and central cells were fused mainly electrically, as shown in Figures 1C at left and 2C. In general, the electrical parameters used in somatic cell fusion could also be used in fusion experiments with sperm and central cells. The sperm–central cell fusion was reproducibly induced by a single pulse or by two to three pulses (0.4 to 0.5 kV $cm^{-1}$). Despite the large difference in cell diameter of the two cell types, the alignment and fusion were made possible by a careful adjustment of the two electrodes. This resulted in a frequency of cell fusion of 44% of 264 sperm–central cell pairs. The cell fusion occurred very rapidly in <1 sec. Most frequently, round sperm cells were used in the fusion experiments. The fusion was most effective and fast when spindle-shaped sperm cells were used and aligned by one of the tail-like ends to the central cell membrane. Under mild hypertonic conditions, reversible contraction of the fertilized central cell occurred immediately after fusion.

Although mechanical alignment was difficult and time consuming (see Figure 1C at right), sperm–central cell fusion in a mannitol solution containing calcium (5 and 10 mM $CaCl_2$) was occasionally observed. Shortly after cell fusion, rapid movement of the sperm nucleus occurred in the central cell cytoplasm, which can be followed light microscopically, as shown in Figure 2E.

### Karyogamy in the Central and Egg Cells

Our technique of nuclei isolation enabled us both to prove the occurrence of karyogamy and to discern the time point of karyogamy by using fluorochromes. At different time intervals after electrofusion, 19 nuclei were isolated from fertilized central cells. After cell fusion, and without karyogamy, secondary nuclei were seen in six samples, and two polar nuclei, positioned close to each other, were observed in three samples, as shown in Figure 4 above the time scale. In these samples, the sperm nucleus was found within the central cell. In one of these samples, adjacent nuclei of a sperm and a secondary nucleus were observed 30 min after sperm–central cell fusion, as shown in Figures 3D and 3E.

The sperm nucleus fused either with the secondary nucleus (six samples) or with one of the polar nuclei (four samples), as demonstrated in Figure 4 below the time scale. The fusion of sperm nuclei with one of the polar nuclei (see Figure 3F) or the secondary nuclei (see Figures 3G and 3H) of the central cell was observed between 1 and 3 hr after cell fusion. One hour after the cell fusion, one nuclear fusion from four examined samples was observed. Unfused sperm

nuclei were also found in the central cell between 1 and 2 hr after cell fusion; however, karyogamy was observed in all samples examined 2 to 3 hr after cell fusion, as shown in Figure 4. Thus, karyogamy happened within 2 hr after central cell fertilization in vitro.

The nucleus isolation procedure also proved to be useful for the rapid determination of the time point of karyogamy in zygotes by removing the cluster of mitochondria and chloroplasts from the vicinity of the egg nucleus. The sperm nucleus was clearly visible in 10 of 20 egg nuclei that were isolated 1 hr after egg–sperm fusion in vitro, as demonstrated in Figures 3I and 3J. In these experiments, the isolated sperm cells were stained with DAPI before cell fusion. Evidently, DAPI staining of sperm cells had no influence on cell fusion, nuclear movement, and karyogamy.

### Development of in Vitro–Produced Endosperm in Culture

Isolated, unfertilized central cells remained alive for up to 1 week in culture. They showed protoplasmic streaming and formed calcofluor white–positive cell wall material around the cells. They were able to form oblong cells and maintained their polarity, indicated by the peripheral position of the organelle cluster. Isolated, unfertilized central cells never developed in culture. However, after in vitro fusion of an isolated sperm with an isolated central cell, the primary endosperm cell elongated very rapidly and developed into structures designated as “in vitro endosperm” in this study. From a total of 40 in vitro–fertilized central cells individually cultivated in inserts (see Figure 1D at left), 19 (48%) developed into multicellular structures.

In the primary endosperm cell, calcoflour white–positive wall material was observed 26 hr after cell fusion around the whole cell, but not inside it, as shown in Figure 5B. During this time, the division of the primary endosperm nucleus was completed, and four nuclei were observed, as demonstrated in Figure 5A. The fertilized central cell, originally round, developed into an oblong structure with a characteristic narrowing. The cell was at the syncytium stage, which could be demonstrated by a careful mechanical manipulation. When the cell was touched with a microcapillary, a flow of cytoplasm from one part into the other and vice versa could be observed without disturbing the shape of the whole cell. At this stage, the division of the primary endosperm nucleus was not followed by wall formation around the nuclei.

In vitro, the endosperm did not develop into callus but rather into a characteristic tissue. Early development of in vitro–produced endosperm showed characteristics comparable to those developed in vivo. The transition from the syncytium to the stage of cellularization was observed 3 to 5 days after in vitro fertilization and is demonstrated in Figures 5C, 5E, 5F, and 5I. Cellularization extended centripetally from the periphery of the primary endosperm cell. In the inner area of the globular part of in vitro–produced endosperm, no cell walls or incomplete cell walls were observed, as demonstrated in Figure 5C. In contrast, in the peripheral regions, cell wall formation had already occurred and uninucleate cells had formed, as shown in Figure 5I. Whereas the mean diameter of the isolated central cell was 203.7 $\pm$ 13.9 $\mu$m, the size of the peripherally located, uninucleate cells of a 4-day-old in vitro–produced endosperm was as small as 38.0 $\pm$ 11.1 $\times$ 34.5 $\pm$ 11.4 $\mu$m, as shown in Figure 5I. One nucleus with a mean diameter of 17.7 $\mu$m and a volume of 2954 $\mu m^3$ was observed in each of these cells. The diameter of the nucleoli in these nuclei was 5.3 $\mu$m.

Intensely fluorescent signals after calcofluor white staining were observed in the globular part rather than in the oblong part, suggesting that cell wall formation extended toward the oblong part of in vitro–produced endosperm. Cell wall formation occurred irregularly and was absent at one pole of the structure (Figure 5E). As shown in Figures 5F and 6A, toward this pole there is a gradient with fewer nuclei, and there are no nuclei at the end of this pole. Most nuclei were observed at the opposite pole.

The reproducibly found oblong structures maintained their polarity and consisted of two parts: one part became globular, containing small cells with dense cytoplasm, and the other part became oblong, ending in large cells at the pole opposite to the globular part of the structure. The oblong part of in vitro–produced endosperm ended in an area containing large, haustorium-like cells, as demonstrated in Figures 5D, 5F, 5G, and 6A. The narrowing was observed at a distinct place (at approximately one-third to one-fourth of the oblong structure) and found in 18 of 19 structures (Figures 5D to 5H).

The polarized oblong multicellular structures developed quickly and reproducibly into structures up to 1120 $\mu$m long 4 days after cell fusion. Five days after in vitro fertilization, the mean length of the cultured structures was 879 $\mu$m, and the mean width was 160 $\mu$m. They reached a size of 1.44 $\times$ 0.72 mm when transferred onto solidified medium 11 days after in vitro fertilization and developed into a white compact tissue, as shown in Figure 5J. In vitro endosperm development was observed after fusion of A188 central cells with sperm cells of line Pirat as well as of line Seneca-60. Cultured on plant regeneration medium, in vitro–produced endosperm did not regenerate roots, shoots, or plants.

### Karyological Analysis

Mitosis and chromosome number were examined in 12 individually Feulgen-stained in vitro–produced endosperm structures. All cells of these structures were uninucleate. After cellularization, cell division apparently became highly synchronized and occurred at a high frequency (Figures 6B and 6D). The analyses revealed $>$25 and up to 30 chromosomes in many cells of the 4- to 6-day-old structures, as demonstrated in Figure 6C. When examined, all structures were predominantly in prophase and only occasionally in the early stages of metaphase and anaphase. Moreover, chromosome

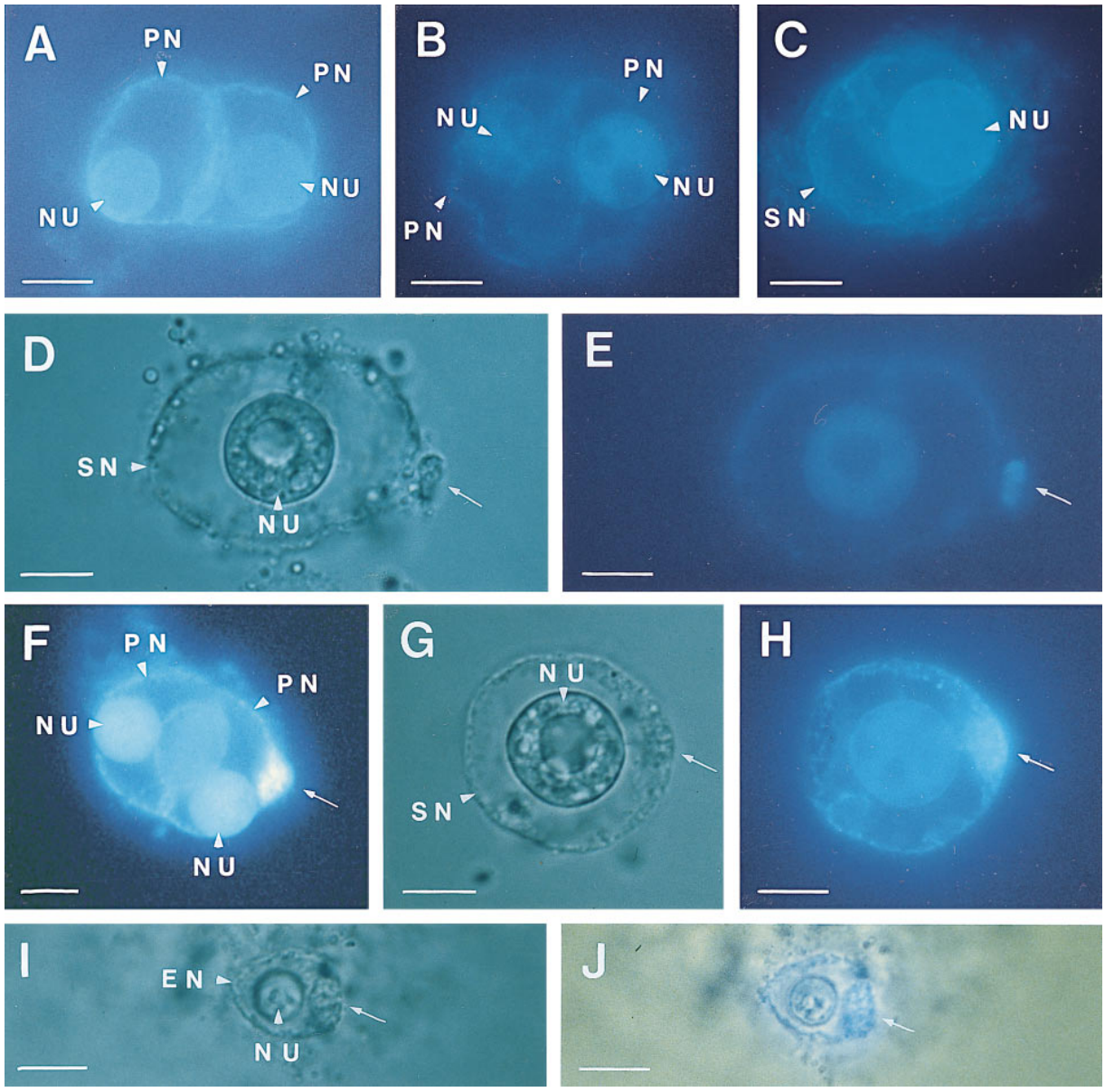


**Figure 3.** Isolated Polar and Secondary Nuclei in Unfertilized Central Cells and Nuclear Fusion in in Vitro–Fertilized Egg and Central Cells in Maize.

Egg and central cell donor line A188 and sperm donor line Pirat were used.
**(A)** Epifluorescence microscopy of two isolated polar nuclei of a central cell positioned close to each other. The central cell was isolated from an unpollinated ovule. Labeled arrowheads indicate the nuclear envelope of each polar nucleus (PN). Each polar nucleus has one large nucleolus (NU). Labeled arrowheads indicate the nucleoli. Bar = 10 μm.
**(B)** Epifluorescence microscopy of two isolated polar nuclei of a central cell positioned close to each other. The central cell was isolated from an unpollinated ovule. Labeled arrowheads indicate the nuclear envelope of each polar nucleus. Each polar nucleus has one large nucleolus with many pores. Bar = 10 μm.
**(C)** Epifluorescence microscopy of an isolated secondary nucleus (SN) of a central cell. The central cell was isolated from an unpollinated ovule. The labeled arrowhead indicates the nuclear envelope of the secondary nucleus. The secondary nucleus has one large nucleolus. Bar = 10 μm.
**(D)** Sperm nucleus (see arrow) attached to the secondary nucleus 30 min after sperm–central cell fusion. A large pore is visible in the nucleolus. Bar = 10 μm.
**(E)** Epifluorescence microscopy of the same sample as shown in **(D)**. The arrow indicates the location of the sperm nucleus. Bar = 10 μm.
**(F)** Epifluorescence microscopy of sperm nucleus (see arrow) before full integration into one of the two polar nuclei, which are positioned close

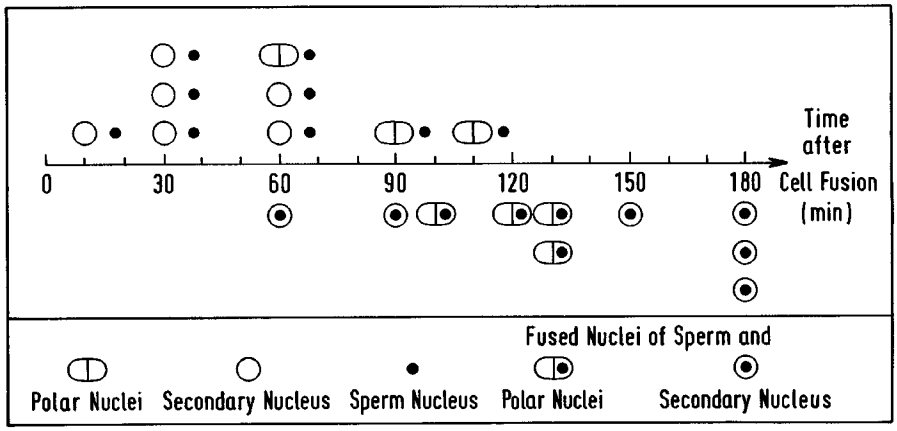


**Figure 4.** Time Course of Karyogamy after Electrofusion of Isolated Maize Central Cells.

Central cell donor line A188 and sperm donor line Pirat were used. After cell fusion and culture, 19 central cells were stained with DAPI at different time points after the isolation of the two polar nuclei, which are positioned close to each other, and secondary nuclei. Nuclei were observed under fluorescent light. In samples without karyogamy, the sperm nucleus was detected within the central cell, as demonstrated above the time scale. Fusion of the sperm nucleus with one of the two polar nuclei or with the secondary nucleus is shown below the time scale.

counting was difficult because of the limited number of samples, the highly synchronous cell division, and the rather poor spreading of the chromosomes by squashing.

## DISCUSSION

The procedure described in this study allowed us to isolate central cells reproducibly. Therefore, it was the basis for the improvement of sperm–central cell fusion experiments and to obtain in vitro–developed endosperm. Our success was due primarily to our increased experience with the manual isolation procedure and the delicate handling of the very large central cell protoplast during all of the other procedures. Also important for central cell isolation was the plasmolysis of the cell before the manual isolation procedure.

Similar to experiments using egg cells or other protoplasts from somatic tissues, not only did the osmolality of the different media play an important role, but so did the shifts from higher to lower osmolality between the steps for central cell isolation, fusion, and culture. As described for sperm–egg fusions (Kranz, 1998), a slight decrease in the osmolality between isolation and fusion (a difference of up to 100 mosmol/kg $H_2O$) to provide slightly hypotonic conditions can be advantageous for efficient sperm–central cell fusion. In the same way, a slight decrease in the osmolality between fusion and culture can help to prevent the floating of unfertilized and fertilized egg and central cells. Sperm–central cell fusion occurred rapidly and efficiently. Using

**Figure 3.** (continued).

to each other, 115 min after sperm–central cell fusion. Each polar nucleus has one large nucleolus. Bar = 10 μm.

**(G)** Isolated secondary nucleus with integrated sperm nucleus (see arrow). Karyogamy occurs 150 min after sperm–central cell fusion. The secondary nucleus has one large nucleolus. A large pore is visible in the nucleolus. Bar = 10 μm.

**(H)** Epifluorescence microscopy of the same sample as shown in **(G)**. The arrow indicates the location of the sperm nucleus inside of the secondary nucleus. Bar = 10 μm.

**(I)** Isolated egg nucleus (EN) with integrated sperm nucleus (see arrow). Karyogamy occurs 1 hr after sperm–egg cell fusion. The labeled arrowhead (EN) indicates the nuclear envelope of the egg nucleus. The labeled arrowhead (NU) indicates the nucleolus of the egg nucleus. Bar = 10 μm.

**(J)** Light and epifluorescence microscopy of the same sample as shown in **(I)**. The arrow indicates the location of the sperm nucleus inside of the egg nucleus. Bar = 10 μm.

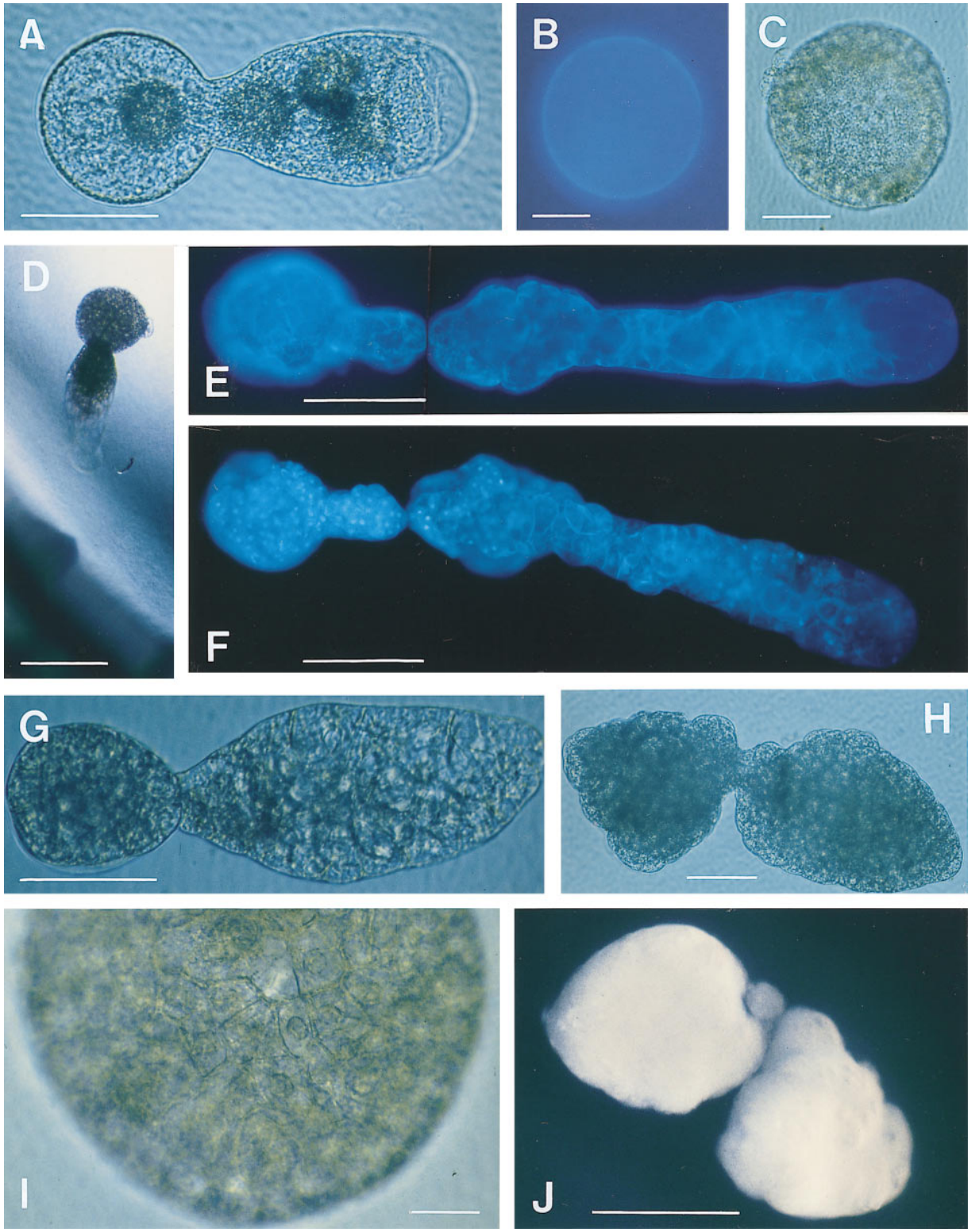


**Figure 5.** Development of in Vitro–Fertilized Maize Central Cells in Culture.

Central cell donor line A188 and sperm donor line Pirat were used.

spindle-shaped sperm cells was most effective, as had been observed in sperm–egg fusions (Kranz et al., 1995). Spindle-shaped sperm cells had been observed during fertilization as early as 1898 (Nawaschin, 1899).

In this study, the fusion of sperm with central cell protoplasts occurred in the presence of calcium ions. Also in maize, the fusion of sperm (Zhang et al., 1997) and the fusion of sperm with egg cell protoplasts have been reported (Faure et al., 1994; Kranz and Lörz, 1994), indicating that calcium ions can promote membrane fusion in generative protoplasts. The involvement of calcium ions in membrane fusion is well known and has been reported earlier in somatic protoplast fusion (e.g., Keller and Melchers, 1973).

For a rapid determination of karyogamy, the method of nuclei isolation after staining of DNA with fluorochromes is simple and much faster than is the time-consuming method of transmission electron microscopy (Faure et al., 1993). Data can be obtained from ~20 to 30 cells in 1 day. We observed both polar and secondary nuclei in isolated and unfertilized central cells, indicating the occurrence of fusion of polar nuclei without pollination and fertilization. However, we observed polar nuclei more frequently than we observed a secondary nucleus in these cells. In vivo, fusion of polar nuclei is accompanied by fusion of the two nucleoli (Vijayaraghavan and Prabhakar, 1984). This is in agreement with our results that indicate the existence of only one large nucleolus in the secondary nuclei. Most of the cell organelles, including large starch grains, are clustered around the nuclei of unfertilized central cells as well as the nuclei of in vitro–produced primary endosperm cells. Similar observations were made with in vivo material of many other species (Vijayaraghavan and Prabhakar, 1984).

After in vitro cell fusion, the fusion of sperm nuclei occurred either with one of the two polar nuclei or with the secondary nucleus. The occurrence of these two types of karyogamy in the isolated central cells is in agreement with results obtained after in vivo pollination and fertilization in maize (Mól et al., 1994) and in contrast with early reports describing the fusion of the sperm nucleus first and exclusively with one of the two polar nuclei, followed by fusion of the polar nuclei (e.g., Rhoades, 1934). By evaluating the in vitro data (this study) of the chronology of karyogamy in egg cells (20 cells) and of Faure et al. (1993; 23 cells examined) in comparison with those in central cells (19 cells examined), we determined that karyogamy occurs in central cells at the same time after gamete fusion or is somewhat delayed when compared with karyogamy in egg cells. As with egg cells, central cell karyogamy is fully completed 2 hr after in vitro gamete fusion.

The elongation of the endosperm began very rapidly, as has been observed in vivo (Randolph, 1936). In vivo, maize endosperm development is described as a nuclear type of endosperm formation (Lopes and Larkins, 1993). We did not observe that karyogenesis and cytokinesis were coupled in the first division of the in vitro–produced primary endosperm cell, as is typical for the helobial type of endosperm development in some monocotyledonous species (Bhatnagar and Sawhney, 1981; Vijayaraghavan and Prabhakar, 1984). However, we observed a narrowing in the in vitro–fertilized, four nuclei–containing endosperm cell, which incompletely divided the cell into two chambers.

In the globular part of the in vitro–produced endosperm, the peripheral cell layer contributed cell layers to its inner part. Thus, cellularization extended centripetally from the periphery, as has been described for in vivo wall formation of nuclear endosperm, which is typical of cereal endosperm, such as maize (Vijayaraghavan and Prabhakar, 1984; Johri et al., 1992; Lopes and Larkins, 1993). Cell wall formation in the nuclear type of endosperm is initiated around the embryo and extends toward the chalazal end (Vijayaraghavan and Prabhakar, 1984). We suggest that the oblong part of our in vitro–produced endosperm represents the chalazal

**Figure 5.** (continued).

**(A)** Primary endosperm cell after 1 day in culture. Bar = 100 μm.
**(B)** Epifluorescence microscopy of a primary endosperm cell after 1 day in culture. The newly formed cell wall is stained with calcofluor white. Bar = 50 μm.
**(C)** Incomplete cellularization in in vitro–produced endosperm after 4 days in culture. The focus is on the middle part of the structure. Bar = 100 μm.
**(D)** In vitro–produced endosperm after 5 days in culture. The fertilized central cell immediately embedded in agarose after in vitro fusion. Bar = 400 μm.
**(E)** Transition from the syncytium to cellularization stage of in vitro–produced endosperm. A composite image of epifluorescence micrographs of cell wall formation inside the cell is shown. Calcofluor white staining of in vitro–produced endosperm after 5 days in culture is shown. Bar = 200 μm.
**(F)** Epifluorescence microscopy of the same structure as shown in **(E)**. Nuclei were stained with DAPI after calcofluor white staining of the cell wall. Bar = 200 μm.
**(G)** In vitro–produced endosperm after 4 days in culture. Bar = 100 μm.
**(H)** Same structure as shown in **(G)** after 6 days in culture. Bar = 100 μm.
**(I)** Uninucleate cells at the periphery of a 4-day-old structure. Bar = 50 μm.
**(J)** Same structure as shown in **(G)** and **(H)** on solid medium after 11 days in culture. Bar = 0.5 mm.

region, because cell wall formation extended toward this pole. Interestingly and typically, the end of the oblong part of the in vitro–produced endosperm consisted of an area of large cells. This group of cells resembles a haustorium. It is similar to that tissue of the nuclear type of in vivo–formed endosperm, which is described as chalazal haustorium (Vijayaraghavan and Prabhakar, 1984).

It has been proposed that the establishment of double fertilization in seed plants resulted in the development of two zygotes per pollen tube, as demonstrated in the nonflowering seed plants *Gnetum gnemon* and *Ephedra* spp, and suggested that endosperm derived evolutionarily from one embryo in the ancestors of angiosperms (Friedman, 1995). The two polar nuclei are genetically identical to the egg nucleus. Therefore, it would be interesting to investigate by using comparative in vitro analyses whether there are similarities in developmental patterns and gene expression programs during, on the one hand, the early development of zygotes and embryos and, on the other hand, the growth of fertilized central cells and endosperm. This might be reflected morphologically by early endosperm development in vitro, which is comparable to the development of the early embryo. Both structures are characterized by a globular part at one pole containing small cells rich in cytoplasm and by an oblong part at the other pole containing large cells (in the embryo, the suspensor cells). We suggest that the globular part of the in vitro endosperm represents the micropylar pole.

In vivo, the endosperm also develops into a heterogenous tissue. In early stages, endosperm increases more rapidly in the basal (micropylar) part than in the apical (antipodal) area (for comparison, see Figures 9E and 9G in Randolph [1936]). Densely cytoplasmic endosperm cells surround the base of the suspensor, whereas large, vacuolated cells surround the maize embryo in other regions (Schel et al., 1984). However, it remains to be determined which part of the in vitro–developed endosperm structure will represent the micropylar pole and which part will represent the chalazal pole. The narrowing of the round, fertilized central cell, which divides two distinct parts of the in vitro endosperm, is maintained during cellularization and further developmental stages. Thus, apart from the feeding effect, the isolated central cell that was fertilized and cultured in vitro without embryo tissue, embryo sac cells, or other maternal tissue is as capable of self-organization as the in vitro–produced zygote is able to self-organize into an embryo without endosperm or any maternal tissue (Kranz and Lörz, 1993). Because this kind of development of the fertilized central cell in vitro was reproducible and typical, this polarization might indicate underlying distinct developmental processes in this unique tissue.

In earlier publications, shoot bud formation in cultured endosperm (Johri and Bhojwani, 1965) and plant regeneration from callus derived from excised and cultured endosperm in some species were reported (reviewed in Bhojwani, 1984; Johri and Rao, 1984). Callus and suspension cultures from excised, immature maize endosperm were established (e.g., Tabata and Motoyoshi, 1965; Shannon and Lui, 1977). Callus cultures did not show regeneration capacity (Straus, 1954), and plant regeneration during culture of maize endosperm explants has not been documented.

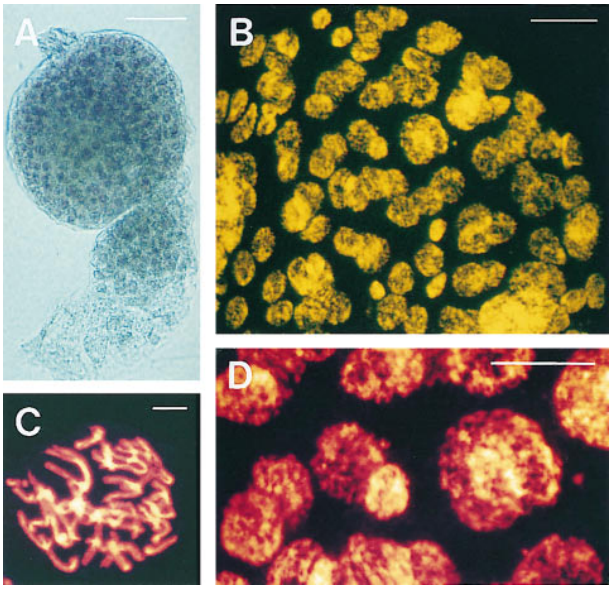


**Figure 6.** Mitoses and Chromosomes in Cells of in Vitro–Produced and Cultured Maize Endosperm.

Central cell donor line A188 and sperm donor line Pirat were used.
**(A)** Feulgen-stained nuclei of in vitro–produced endosperm after 5 days in culture. Bar = 100 μm.
**(B)** Synchronous cell division. Shown is a three-dimensional survey of a section of in vitro–produced endosperm after 4 days in culture. Bar = 20 μm.
**(C)** Advanced prophase of 30 chromosomes of in vitro–produced endosperm after 4 days in culture. A series of nine single sections was used to create this image. Bar = 5 μm.
**(D)** Synchronous cell division in in vitro–produced endosperm after 4 days in culture: early prophase. Bar = 5 μm.

Also, we did not observe root or shoot regeneration in structures that were derived from cultured, in vitro–fertilized central cells. It remains to be determined whether in vitro–produced endosperm can be maintained in long-term cultures as a specific tissue or whether it develops and establishes other properties in culture. Also, we need to determine whether the in vitro–produced endosperm is able to regenerate roots, shoots, and plants in culture.

Rapid and highly synchronous cell divisions were observed during early development of our in vitro–produced endosperm, as was found in vivo in maize (Kowles and Phillips, 1988). In vivo, mitotic activity in maize endosperm reaches a peak between 8 and 10 DAP. Nearly no mitoses were observed between 12 and 14 DAP, and the process of endoreduplication was initiated between 10 and 12 DAP (Kowles and Phillips, 1988; Grafi and Larkins, 1995). In the basically triploid endosperm, mitotic inhibition occurs at prophase or

metaphase (Vijayaraghavan and Prabhakar, 1984). Interestingly, in this respect, we found predominantly prophases in all 4- to 6-day-old in vitro–produced endosperms.

After the egg–sperm in vitro fusion, the diploid set of chromosomes ($n = 20$) was found in hybrid maize plants by using the combination line A188 (egg donor) and line Pirat (sperm donor) (Kranz and Lörz, 1993). Using the same combination of lines in the central cell–sperm fusion in this study, the observed chromosome numbers of 25 to 30 indicate the triploid nature of in vitro–produced endosperm.

In summary, central cell fertilization is reproducibly possible in vitro. (1) Sperm–central cell fusion occurs very quickly. (2) Isolation of nuclei is a useful method for rapid determination of karyogamy in central and egg cells. (3) Karyogamy occurs in the isolated central cell after fusion with a sperm cell. (4) Fusion of sperm nuclei occurred with one of the two polar nuclei or with the secondary nucleus and is completed within 2 hr after cell fusion. (5) Unfertilized central cells do not divide in culture. However, in vitro–produced endosperm develops in culture and not simply as a callus. The isolated, in vitro–fertilized central cell develops in a predictable way independently of the zygote and of mother tissue and shows characteristics comparable to the situation in vivo.

Isolated central cells can now be used for studies of surface molecules. It will be of particular interest to study whether there exists a specificity with putative sperm receptor molecules. In addition, a recognition assay might be developed to test specificity of receptors in adhesion and fusion experiments. The technique developed in this study might be used to investigate especially early events of endosperm formation. Early signal transduction events, the patterns of mitotic divisions, changes in the cytoskeleton, nuclei migration and positioning, the timing and characteristics of cell wall formation, and the patterns of the cells formed can now be studied in the fertilized central cell under more defined conditions. Underlying mechanisms of programming of nuclear location and of division planes in endosperm during syncytium and cell wall formation have not been investigated to date (Walbot, 1994). Moreover, comparable in vitro conditions allow a comparison of events like these with the development of the in vitro zygote.

Endosperm development without fertilization occurs in a mutant of Arabidopsis (Ohad et al., 1996). Similar to isolated unfertilized egg cells, the central cells did not divide under our standard culture conditions. It is feasible that cell divisions can be triggered in central cells by modifications of the culture medium (for example, by a short treatment with high amounts of 2,4-D), as has been done with unfertilized cultured egg cells (Kranz et al., 1995).

Endosperm development can now be studied by defined modifications of in vitro conditions, especially by modifications in media composition. To facilitate the elucidation of the role of the fertilized central cell during embryogenesis, it might also be interesting to analyze substances secreted by the developing endosperm into the culture medium. Moreover, such substances might have a growth-promoting influence on embryo development by coculture of in vitro–fertilized egg and central cells.

With the possibility of in vitro fusions with more than one sperm cell, experiments with central cells can be designed to investigate dosage effects and imprinting. cDNA libraries from a few unfertilized and fertilized central cells can now be constructed for gene isolation, as has been done with maize egg cells and in vitro–developed zygotes (Dresselhaus et al., 1994, 1996), to obtain detailed information on molecular processes of early development (Kranz and Dresselhaus, 1996). Thus, our technique developed for in vitro central cell fertilization will be useful for further studies on early endosperm development. These studies will help us to gain more insight into the coordinated development of both the egg and the central cell. In this respect, detailed information on the regulation of the first cell cycle in the zygote and also in the fertilized central cell is needed (Sauter et al., 1998). These investigations using cell and molecular biological methods to study single cells may contribute to a better understanding of double fertilization and early seed development.

## METHODS

### Plant Material

Maize (*Zea mays*) inbred line A188 (courtesy of A. Pryor, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) was used for egg and central cell isolation, and the commercial hybrid line Pirat and line Seneca-60 (courtesy of M. Schwall, Südwestdeutsche Saatzucht, Rastatt, Germany) were used for sperm cell isolation. The plants were grown in the greenhouse under standard conditions.

### Isolation and Selection of Sperm and Central Cells

Collection and storage of pollen as well as the isolation of sperm cells were performed as previously described (Kranz et al., 1991a). For central cell isolation, ovules were selected from the middle part of ears (emerged silk length, 3 to 18 cm; ovule diameter, 2 to 3 mm), which were bagged before silk emergence. Before ovule selection, the outer leaves of the ears were surface sterilized with ethanol (70%). Nucellar tissue pieces were dissected from the ovules under a dissecting microscope. Approximately 30 tissue pieces containing the embryo sac were collected in a 1-mL mannitol solution (osmolality of 750 mosmol/kg $H_2O$ for culture experiments of unfertilized and fertilized central cells and of 850 mosmol/kg $H_2O$ for nuclei isolation experiments, respectively) in 3-cm-diameter plastic dishes, followed by the addition of 0.5 mL of a mannitol solution (570 mosmol/kg $H_2O$) containing a mixture of the following cell wall–degrading enzymes: 1.5% pectinase (Serva, Heidelberg, Germany), 0.5% pectolyase Y23 (Seishin, Tokyo, Japan), 1.0% cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), and 1.0% hemicellulase (Sigma), pH 5.0. Incubation was at room temperature for 45 min to 1 hr without shaking.

After this treatment, central cells were manually isolated directly in the incubation dish with glass needles under an inverted microscope

(Axiovert 135; Carl Zeiss, Oberkochen, Germany). Some nucellus cells were removed only from the micropylar end near the embryo sac. Beginning at the chalazal end, near the antipodal cells, the central cell was pushed toward the micropylar end with a glass needle. Here, the cell was liberated and became spherical. Occasionally, it was possible to isolate a unit of an egg, two synergids, and a central cell. Subsequently, the adherent egg and synergids were removed manually from the central cell by using a microneedle.

Sperm cells were isolated after bursting of mature pollen grains by osmotic shock in mannitol solution. The osmolality of this solution was 650 mosmol/kg $H_2O$ when culture experiments were performed and 800 mosmol/kg $H_2O$ for nuclei isolation experiments. For egg cell isolation, ~30 nucellar tissue pieces were collected in 1 mL of mannitol solution (650 mosmol/kg $H_2O$) in 3-cm-diameter plastic dishes, followed by the addition of 0.5 mL of enzyme solution, as described above. Incubation was at room temperature for 30 min without shaking and was followed by manual dissection. For sperm–egg fusions, sperm cells were isolated in mannitol solution (650 mosmol/kg $H_2O$).

Sperm, central, and egg cells were selected under a microscope and transferred by microcapillaries by using a computer-controlled micropump (dispenser/diluter, Microlab-M; Hamilton, Darmstadt, Germany), as described by Koop and Schweiger (1985a) and Kranz et al. (1991a). Central cells were transferred into another microdroplet for washing (mannitol solution, 650 and 800 mosmol/kg $H_2O$, respectively). For sperm selection, microcapillaries with a tip opening of 20 μm were used. Central cells were individually transferred by microcapillaries with a tip opening of 300 μm into the microdroplets. Capillaries with tip openings of 200 μm were used for egg cell selection and transfer.

### Fusion of Sperm and Central Cells

Fusion of a sperm with a central cell was performed mainly electrically, as described previously by Kranz et al. (1991a, 1991b) and Kranz and Lörz (1993), using the technique of Koop and Schweiger (1985b), or chemically in a calcium-containing medium (Faure et al., 1994; Kranz and Lörz, 1994).

Individual electrofusion was performed using microelectrodes of platinum fixed to a support, which was mounted under the condensor of the microscope, and an electrofusion apparatus (CFA 400; Krüss, Hamburg, Germany). Alignment, adhesion, and fusion of the cells were continuously observed under an inverted microscope (IM 35; Carl Zeiss). The fusion medium consisted of mannitol solution (650 mosmol/kg $H_2O$ in culture experiments and 800 mosmol/kg $H_2O$ in nuclei isolation experiments, respectively). Generally, the osmolality of the fusion medium was lower (50 or 100 mosmol/kg $H_2O$) than that of the isolation medium.

The electrical conditions for dielectrophoretic alignment were 1 MHz and 38 to 56 V $cm^{-1}$. Cell fusion was induced by a single pulse or by two to three negative direct current pulses (50 μsec; 0.4 to 0.5 kV $cm^{-1}$). Egg–sperm fusions were induced by a single pulse or two to three negative direct current pulses (50 μsec; 0.9 to 1.0 kV $cm^{-1}$) after dielectrophoretic alignment (1 MHz; 70 V $cm^{-1}$) on one of the microelectrodes. Egg–sperm fusions were performed in microdroplets of mannitol (600 or 650 mosmol/kg $H_2O$).

Chemical fusion was performed manually using a microneedle for alignment of the two cells and a calcium-containing (5 and 10 mM $CaCl_2$) mannitol solution (600 to 650 mosmol/kg $H_2O$), pH 6.0. After the fusion, the fertilized central cells were transferred into the microcapillary by several suction steps of each 15 nL and gently released onto the bottom of the culture dish.

### Culture Procedures

Unfertilized and in vitro–fertilized central cells were cultured as described previously for in vitro zygotes (Kranz et al., 1991a; Kranz, 1998) but with minor modifications. Briefly, the fusion products were cultured in inserts (12-mm-diameter Millicell-CM dishes; Millipore, Bedford, MA) that had been filled with 100 μL of medium. These dishes were located in a 3.5-cm-diameter plastic dish filled with 1.5 mL of a maize feeder suspension (Kranz et al., 1991a). For culture, a modified Murashige and Skoog medium (Murashige and Skoog, 1962) was used as described by Kranz and Lörz (1993). In some experiments, to prevent bursting of floating cells on the surface of the medium, unfertilized and fertilized central cells were transferred onto the membrane of a Millicell-CM dish that had been filled with 100 μL of a semisolidified mannitol solution (600 mosmol/kg $H_2O$; 0.5 and 0.75%, respectively, ultralow gelling temperature agarose, type IX; Sigma). The cultures were maintained on a rotary shaker at 50 rpm, starting 6 days after cell fusion. The culture conditions were 26 ± 1°C, a light/dark cycle of 16/8 hr, and a light intensity of ~50 μmol $m^{-2}$ $sec^{-1}$.

Six days after the cell fusion, the insert containing the structures was transferred into one well of a four-well multidish (Nunclon; Nunc A/S, Roskilde, Denmark) that had been filled with 300 μL of the previous conditioned medium but without feeder cells. When the structures reached a length of ~1.5 mm and a width of ~0.7 mm (that is, ~11 days after in vitro fertilization), they were subcultured on solidified 2.0 mL of modified Murashige and Skoog medium containing 60 g/L sucrose, no hormones, and 4 g/L agarose (type I-A; Sigma) in a 3.5-cm-diameter plastic dish (Kranz and Lörz, 1993) and maintained under the culture conditions described above.

### Isolation of Nuclei

Nuclei of in vitro–produced zygotes, of central cells, and of fertilized central cells were isolated in a modified isolation buffer (Schweizer et al., 1989) containing 20 mM Tris, pH 7.8, 5 mM $MgCl_2$, 5 mM KCl, and 0.5% (v/v) Triton X-100. The isolation of nuclei was performed manually under continuous microscopic observation in 2000-nL droplets covered by mineral oil by using a glass needle to squash the cell and to remove the remaining cell material from the nuclei. Isolated nuclei were washed three times in the microdroplets with 1000 nL of isolation medium.

### Chromosome Staining

Chromosomes were stained using the Feulgen procedure. Basically, the single steps were performed in a Millicell-CM dish (12 mm in diameter) containing a structure in 100 μL of the appropriate solution. This dish was inserted in 3-cm-diameter plastic dishes filled with 2 mL of the same solution. The structures were transferred individually from one solution to the next by using a microcapillary connected to a micropump. Briefly, individual and fast-growing structures in the Millicell-CM dish and feeder cells were treated for 3 to 19 hr in the dark with colchicine dissolved in growth medium. One hundred microliters

from a colchicine stock solution (5%) was mixed with 2 mL of conditioned growth medium of the preculture. Three hundred microliters of this solution was added to the Millicell. The insert was transferred into a 3-cm-diameter plastic dish that had been filled with the remaining colchicine-containing solution (1700 μL) and with feeder cells from the preculture. After overnight fixation in ethanol–glacial acetic acid (3:1) and successive incubation in ethanol (50%), ethanol (25%), and double-distilled water (each 10 min), the samples were treated with HCl (1 M) for 12 min at 60°C, washed with double-distilled water, and treated with Schiff's reagent (Sigma) for 1 hr.

After washing, the structures were treated with sodium disulfite (5%), washed, and squashed. In some experiments, and before squashing, single structures were treated with a mixture of cell wall–degrading enzymes (1.5% pectinase, 0.5% pectolyase Y23, 1.0% cellulase Onozuka RS, and 1.0% hemicellulase) dissolved in mannitol solution (570 mosmol/kg $H_2O$), pH 5.0. For this treatment, the structures were transferred individually into a droplet (100 μL) of enzyme mixture previously placed onto a microscope slide and incubated for 30 min at room temperature in a humid chamber. After washing and mechanical separation of the tissue with a microneedle under a microscope, single cells were easily obtained and distributed in the droplet for chromosome counting by using a laser scanning microscope.

### Laser Scanning Microscopy

Mitotic phases and chromosome numbers of Feulgen-stained in vitro–produced endosperm tissue were examined using a confocal laser scanning microscope (TCS-4D; Leica, Heidelberg, Germany) equipped with a krypton–argon laser. Excitation was at 520 to 580 nm, and fluorescence light detection was at wave lengths >620 nm. Images of several optical sections in the tissue were collected, scrutinized, and stored for later documentation on Ektachrome 100 films (Kodak).

### Fluorescence Microscopy

In general, the procedures were performed under a microscope on a coverslip in microdroplets, which were covered by mineral oil. Newly formed cell wall material was stained with calcofluor white ST (American Cyanamid Company, Bound Brook, NJ). One hundred nanoliters of a stock solution (1 mg/mL in mannitol solution; 800 mosmol/kg $H_2O$) was added to the 2000-nL microdroplet of mannitol solution (800 mosmol/kg $H_2O$) containing 10 μM $CaCl_2$ or nutrient solution, respectively, and the cell. A 10-min incubation was followed by three washings with 1000 nL of mannitol solution.

DNA of isolated egg and central cell nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). One hundred nanoliters of a stock solution (1 mg/mL in mannitol solution; 800 mosmol/kg $H_2O$) was added to the 2000-nL droplet of nuclei isolation solution. The nuclei were stained for 10 min and then washed three times with nuclei isolation solution. In another set of experiments, the sperm cells were stained during isolation (100 μL of DAPI stock solution was added to 4 mL of pollen suspension). Released sperm cells were selected and washed twice in microdroplets of mannitol solution before cell fusion. Individually, stained cells and nuclei were examined using an epifluorescence inverted microscope (Axiovert 35M, filter set No. 01; Carl Zeiss). Fluorescence micrographs were recorded on Ektachrome 64T (EPY 135; ISO 64) films.

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