

Karyogamy after Electrofusion of Single Egg and Sperm Cell Protoplasts from Maize: Cytological Evidence and Time Course

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In maize, *in vitro* fusion of isolated male gametes with isolated egg cell protoplasts can be induced by electric pulses. Until now, karyogamy has not been demonstrated. In this study, we cytologically examined fusion products fixed at different times after electrofusion with phase contrast microscopy and transmission electron microscopy. We obtained a precise timetable from 23 samples studied during the first 3 hr. The sperm nucleus was integrated within the egg cell protoplast, migrated toward the egg cell nucleus, and fused with it within 1 hr, as demonstrated by ultrastructural observations, three-dimensional reconstructions of nuclei, and subsequent nuclear volume estimates. Fusion of nuclei occurred before zygotic mitosis, as is the case *in vivo*. These findings demonstrate karyogamy during *in vitro* fertilization of maize.

INTRODUCTION

The fertilization of egg and central cells, which are deeply enclosed in maternal tissues, by male gametes issued from the pollen tube was discovered in flowering plants approximately one century ago (Nawaschin, 1898; Guignard, 1899) and termed "double fertilization." However, the mechanisms underlying this process are still largely unknown in comparison with fertilization in animals. Several questions remain unanswered, such as the mechanisms of a possible intergametic recognition, the precise process of gametic fusion, the block to polyspermy, and the early metabolism of the zygote following fertilization. Our lack of knowledge is due mainly to the inaccessibility of the gametes. Hence, the performance of fertilization *in vitro* should allow detailed study of the fertilization event under controlled and accessible conditions, as has been done in animals (Dale, 1991).

Maize is a suitable plant with which to perform *in vitro* fertilization. Many genetic and cytogenetic analyses have been conducted (Sheridan, 1988), and the partners involved in fertilization have been well studied, as will be presented by Dumas and Mogensen (1993). In particular, both male (Dupuis et al., 1987; Cass and Fabi, 1988) and female (Wagner et al., 1989a) gametes have been isolated from maize, and the first *in vitro* fusion of an isolated egg cell with an isolated sperm cell by electrofusion was performed in maize (Kranz et al., 1990, 1991). However, as discussed by Chasan (1992), nuclear fusion

following the cytoplasmic fusion remains to be demonstrated, and regeneration of plants from the fusion products has yet to be performed. Embryogenesis and regeneration of fertile plants are now possible (Kranz and Lörz, 1993). In this article, we describe the fate of the male nucleus after electrofusion and demonstrate the occurrence of complete karyogamy.

To follow the fate of the male nucleus, we have chosen transmission electron microscopy, which is a more precise tool to follow small nuclei than is fluorescence or confocal laser scanning microscopy and which allows complementary ultrastructural observations. We conducted the fixation of cells at precise time intervals after electrofusion.

RESULTS

Penetration of the Male Nucleus into the Egg Cell Protoplast

We used the general procedure described in Figure 1 for the study of electrofusion products from isolated male and female gametes. In each of three samples fixed 7 to 8 min after fusion, a dark object of $\sim 4 \mu\text{m}$ in diameter was observed on semithin sections, as shown in Figure 2A. We interpreted this object to be the male nucleus. We confirmed this analysis with transmission electron microscopy and by reembedding and ultrathin sectioning the semithin sections containing the

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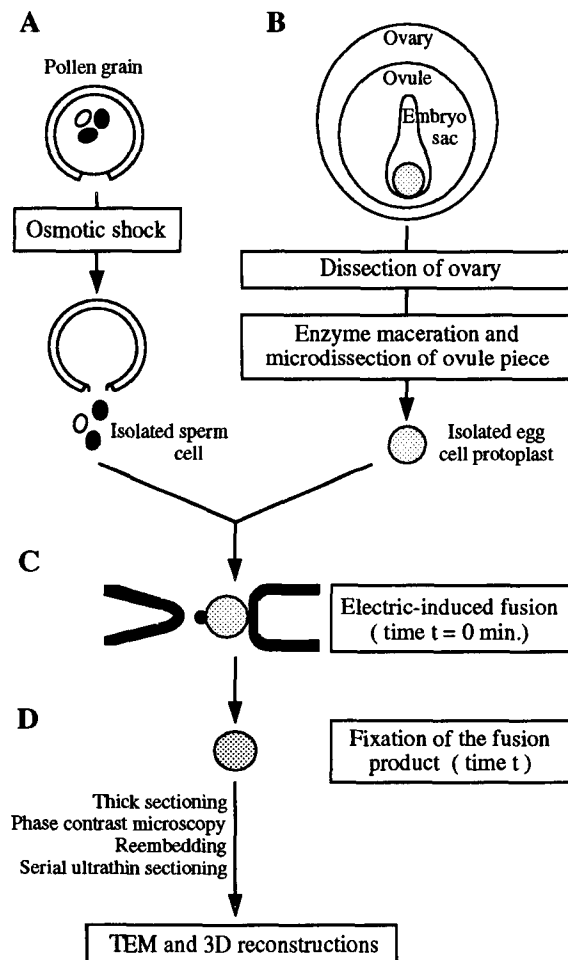


Figure 1. General Procedure for the Study of Electrofusion Products from Isolated Male and Female Gametes in Maize.

(A) Isolation of male gametes from pollen grains by osmotic shock in 0.5 M mannitol.

(B) Isolation of egg cell protoplasts. Ovule pieces containing the egg cells within embryo sacs were removed from ovaries by dissection, incubated with enzymes, and microdissected.

(C) Electric-induced fusion of the isolated gametes after their alignment by dielectrophoresis.

(D) Fixation of the fusion products at different times (t) from 7 min to 3 hr after electrofusion ($t = 0$ min) and treatment for transmission electron microscopy (TEM) and three-dimensional (3D) reconstructions.

putative male nuclei. Figure 2B shows that the male nucleus has entered the egg cell protoplast. The male nucleus has a more condensed chromatin than that of the egg cell nucleus. It has no nucleolus and has a circular profile in section view (Figure 2B). At this time, the male plasma membrane is no longer present. We also observed the overall structure of the fusion products with transmission electron microscopy. The cytoplasm contains numerous amyloplasts and mitochondria

close to the spherical euchromatic female nucleus. This cluster of organelles is surrounded by vacuoles, and no cell wall components were observed around the fusion products.

Thus, in all of the samples fixed within the first 10 min after fusion, the sperm nucleus was integrated in the egg cell cytoplasm. However, in two samples, this stage was still observed later: in one at 30 min and in one at 105 min (Figure 2C) after the pulses.

Adjacent Male and Female Nuclei

We observed five samples with two apposed nuclei using transmission electron microscopy, all fixed between 10 and 55 min after electrofusion: Figure 2D shows a 21-min sample and Figure 2E shows one collected at 30 min after electrofusion. The smallest nucleus and its envelope are closely apposed to the female nucleus, as observed in Figure 2E. However, the smallest nucleus is not connected to the female nucleus, as seen in serial sections from the five samples and as shown in Figure 3A, which is a three-dimensional reconstruction 21 min after electrofusion. We identified this nucleus as the male nucleus because of its high chromatin density and its estimated volume ($55 \mu\text{m}^3$), which is very similar to the nuclear volume of an isolated sperm cell. Although the male nucleus is surrounded by some small mitochondria with highly contrasted cristae (Figure 2E), we have not been able to clearly identify mitochondria originating from the male gamete.

Fusion of Male and Female Nuclei

Inside each of four protoplasts fixed between 45 and 60 min after electrofusion, it was possible to detect only one nucleus in phase contrast microscopy. Each of these nuclei had a darker area on the side, as shown in Figure 4A. We reembedded and thin sectioned the pertinent thick sections of the four samples to determine their nuclear structure using transmission electron microscopy. The dark area, shown in Figure 4B, corresponds to a side expansion of the nucleus with denser chromatin 58 min after the electric pulses. This denser chromatin is similar to chromatin of male nuclei within early electrofusion products, as shown in Figures 2B to 2E. The expansion has the shape of a side bud, as shown in Figure 4B and in a three-dimensional reconstruction in Figure 3B. In addition, a remnant of the nuclear envelope was observed in a few serial sections between the dense and the lighter chromatin, as shown in Figure 4B. Thus, we interpreted these results from four samples as the fusion event of male nuclei with female nuclei. A second three-dimensional reconstruction with a demarcation between dense and lighter chromatin, shown in Figure 3C, allowed us to estimate the volume of male chromatin that was still identifiable. The estimated volume ($134 \mu\text{m}^3$) is $\sim 140\%$ higher than before nuclear fusion. Thus, male chromatin probably becomes less condensed as the fusion with the female nucleus takes place.

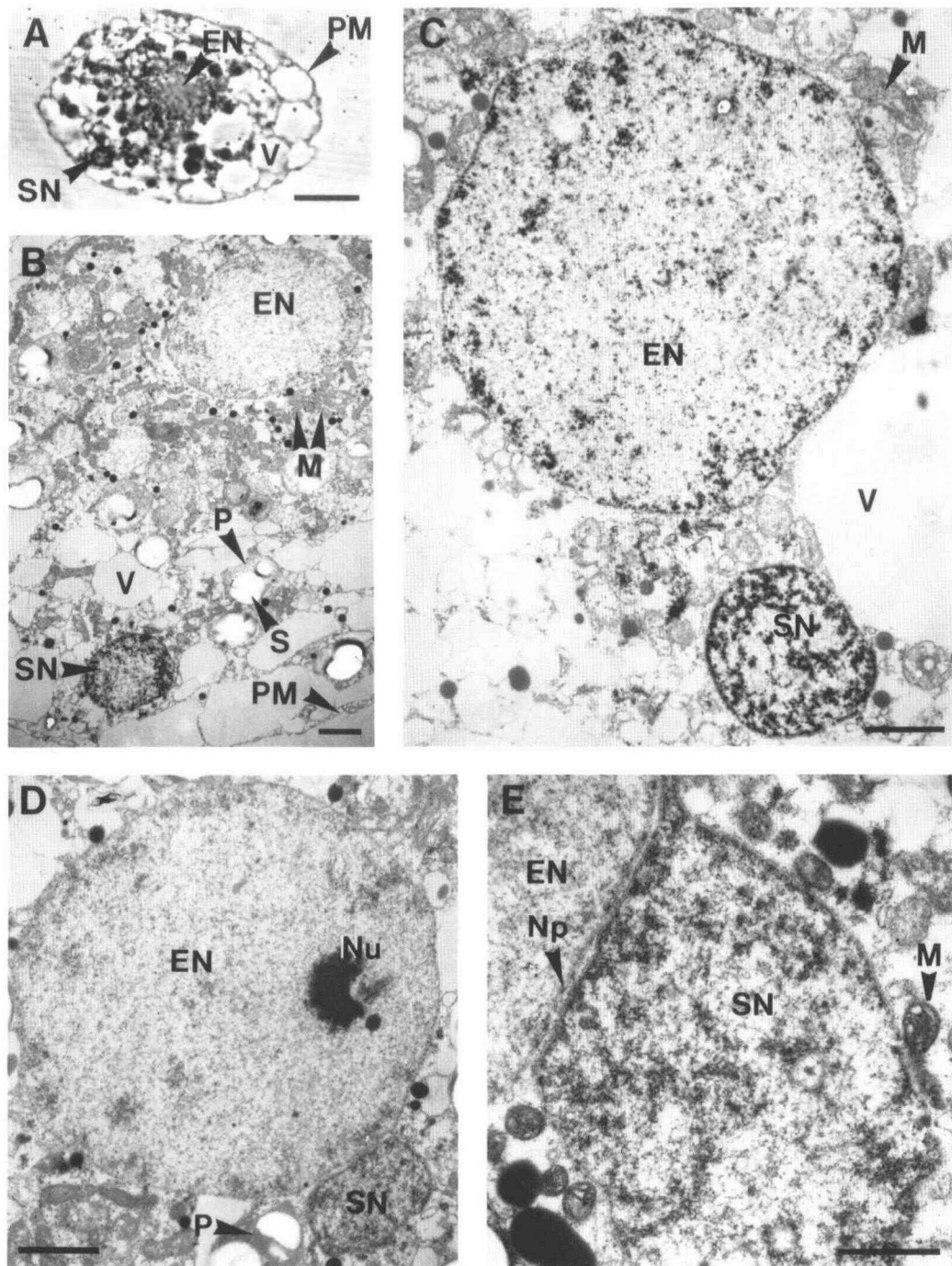


Figure 2. Sperm Nucleus within the Egg Cell Cytoplasm after Electrofusion of Isolated Maize Gametes.

(A) Phase contrast micrograph of a thick section of an electrofusion product fixed 8 min after the electric pulses. Labeled arrowhead (SN) indicates the sperm nucleus. The cytoplasm also contains numerous peripheral vacuoles (V) and a central aggregation of organelles with the edge of the egg cell nucleus (EN). PM, plasma membrane. Bar = 10 μ m.

(B) Electron micrograph of an ultrathin section taken from the same section as shown in **(A)** with a sperm nucleus (SN) identifiable by its dense chromatin inside the egg cell protoplast with the egg cell nucleus (EN). M, mitochondria; P, plastid; PM, plasma membrane; S, starch grain; V, vacuole. Bar = 4 μ m.

(C) Electron micrograph of an electrofusion product fixed 105 min after the electric pulses with a dense sperm nucleus (SN) and the egg cell nucleus (EN). M, mitochondrion; V, vacuole. Bar = 2 μ m.

(D) Electron micrograph of apposed sperm nucleus (SN) and egg cell nucleus (EN) within an electrofusion product fixed 21 min after the electric pulses. No connection between the two nuclei was observed in serial sections. P, plastids; Nu, egg cell nucleolus. Bar = 2 μ m.

(E) Electron micrograph of an electrofusion product fixed 30 min after the electric pulses with apposed sperm nucleus (SN) and egg cell nucleus (EN). Nuclear envelopes with nucleopores (Np) are very close together but not connected. Note the presence of small mitochondria (M) with contrasted cristae along the male nuclear envelope. Bar = 1 μ m.

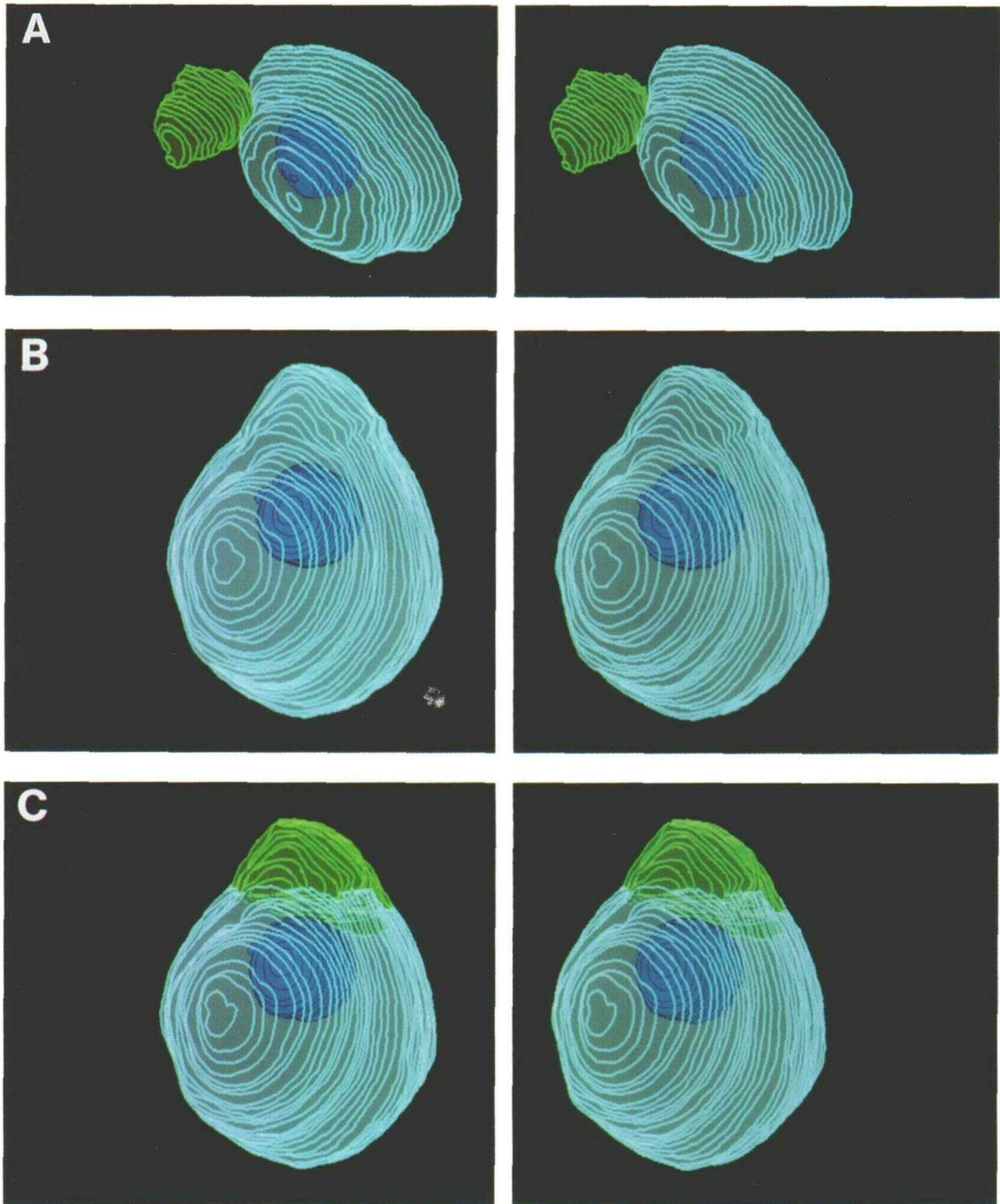


Figure 3. Stereoscopic Pairs of Reconstructed Nuclei in Electrofusion Products.

(A) Reconstruction of nuclei 21 min after electrofusion. The sperm cell nucleus (yellow-green), the egg cell nucleus (light blue), and the egg cell nucleolus (dark blue) are outlined in every section. The sample used for the three-dimensional reconstruction is shown in Figure 2D. Approximate magnification $\times 2610$.

(B) Reconstruction of the nucleus (light blue) with its nucleolus (dark blue) in every section 58 min after electrofusion. Note the budlike shape in the upper part of the nucleus, which corresponds to the fusing sperm nucleus. The sample used for the three-dimensional reconstruction is shown in Figures 4A and 4B. Approximate magnification $\times 3130$.

(C) Reconstruction 58 min after electrofusion from the same sample shown in **(B)**. Additional outlines have been used (yellow-green) to represent the male chromatin, which is still identifiable. Approximate magnification $\times 3130$.

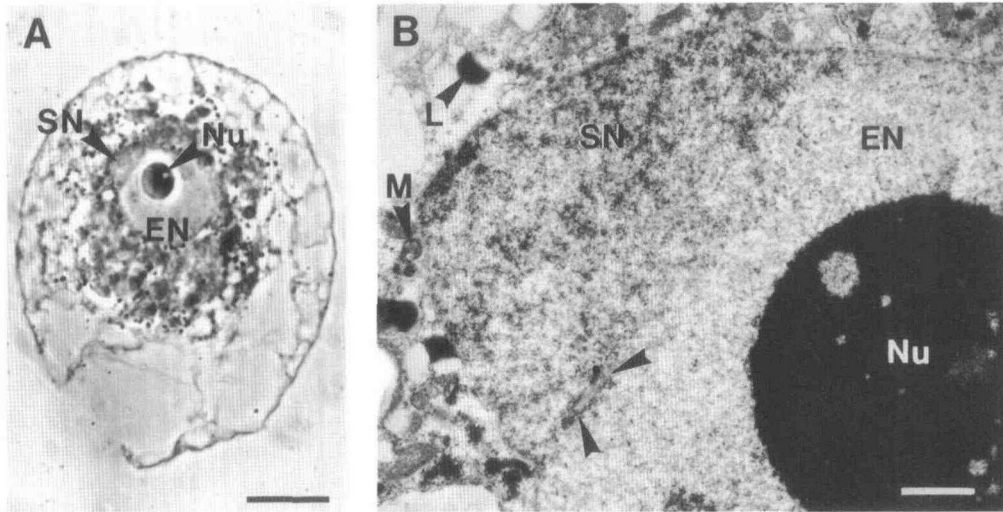


Figure 4. Sperm Nucleus Fusing with the Egg Cell Nucleus after Electrofusion of Isolated Maize Gametes.

(A) Phase contrast micrograph of a thick section from a sample fixed 58 min after electrofusion. The sperm nucleus (SN) is fusing with the egg cell nucleus (EN). Nu, egg cell nucleolus. Bar = 10 μm.

(B) Electron micrograph of an ultrathin section taken from the same section shown in (A) depicting the fusion of the sperm nucleus (SN) with the egg cell nucleus (EN). Note the difference in chromatin density and a nuclear envelope remnant (unlabeled arrowheads) close to the male chromatin. L, lipid body; M, mitochondrion; Nu, egg cell nucleolus. Bar = 1 μm.

In the 10 samples fixed later, between 60 and 195 min after electrofusion, with one exception (already described in Figure 2C), only one nucleus was observed in each protoplast by means of phase contrast microscopy. In five of these samples, the unique nucleus contained two nucleoli.

shown in Figure 5. The male nucleus penetrated the cytoplasm of the egg cell protoplast within the first 20 min, became apposed to the female nucleus between 20 and 45 min, fused with it between 45 and 60 min, and was integrated into the egg nucleus after 60 min.

Chronology

Results from the 23 samples described above, all of which were examined within the first 3 hr after electrofusion, were quite consistent, thereby enabling us to establish the chronology

DISCUSSION

The following observations indicated that karyogamy occurs after the electrofusion of isolated maize gametes. (1) Both

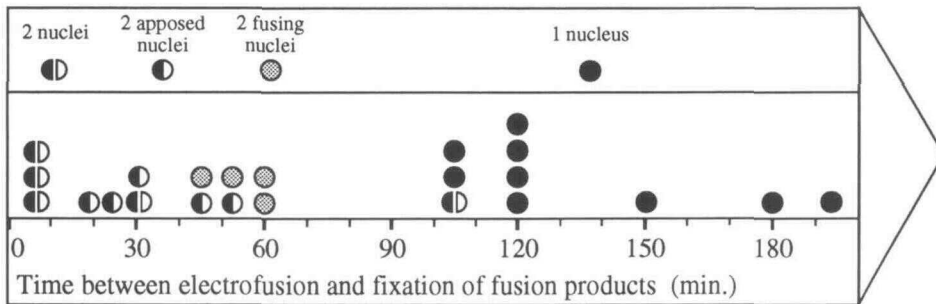


Figure 5. Chronology of the Fate of Male and Female Nuclei after the Electrofusion of Isolated Maize Gametes.

Each of the 23 samples studied is put on a time scale and is symbolized by one of the four different patterns described in the upper part of the figure. Patterns are given according to nuclear stages, i.e., two nuclei, two apposed nuclei, two fusing nuclei, or only one nucleus.

closely apposed and fusing male and female nuclei were visible with transmission electron microscopy (Figures 2 and 4). Male and female nuclei were identified by comparing their morphology with those of isolated sperm cells (Wagner et al., 1989b; Mogensen et al., 1990) and nonfertilized isolated egg cell protoplasts (Faure et al., 1992). In addition, the volume of male chromatin estimated from three-dimensional reconstructions (Figure 3) was in agreement with that of isolated sperm cells (Mogensen et al., 1990). (2) A consistent chronology was obtained when the results from the 23 samples fixed at different times after electrofusion were compiled (Figure 5).

Time Course of Karyogamy

The time course from plasmogamy to karyogamy is difficult to estimate *in vivo*. Indeed, the fusion of a sperm cell with a central cell was shown once in *Plumbago zeylanica* (Russell, 1983) with transmission electron microscopy, but, to our knowledge, fusion between egg and sperm cells has never been shown at this level. Therefore, there is no precise reference time *in vivo*. Nevertheless, some estimates have been published in which male gametes have been observed close to egg cells or male nuclei have been seen within the egg cell cytoplasm after gametic fusion. Karyogamy seems to occur within ~1 to 2 hr after plasmogamy in cereals, such as wheat (You and Jensen, 1985; Guo-Wei and Jia-Heng, 1992), Triticale (Hause and Schröder, 1987), and probably barley (Mogensen, 1982). No data are available in maize, although up to 22 hr may elapse between pollination and fertilization, depending on silk length and environmental conditions (Diboll, 1968; R. Mol, E. Matthys-Rochon, and C. Dumas, manuscript in preparation).

By comparison with *in vivo* studies, we have obtained a precise timetable *in vitro*, in which the time of gametic fusion is well defined and controlled. Karyogamy occurs 45 to 60 min after gametic fusion under our experimental conditions. Therefore, maize electrofusion products appear to behave like zygotes obtained *in vivo* in other grasses with regard to the time course of karyogamy.

Fate of the Male Nucleus

The male nucleus first migrates toward the female nucleus, as it does in animals (for review, see Longo, 1988). The use of colcemid, an inhibiting factor of microtubule formation, prevents pronuclear movement in sea urchin (Zimmerman and Zimmerman, 1967), rabbit (Longo, 1976), and mouse (Schatten et al., 1985). Therefore, microtubules are apparently involved in the movement of the male pronucleus and its apposition to the female pronucleus. However, the mechanism of this migration is not known and is highly speculative in animals (for review, see Longo, 1988). Microtubules have been detected by immunocytochemistry in the mature egg cells of some

higher plants (see Huang and Russell, 1992), but to our knowledge, no study has demonstrated their involvement in nuclear migration. The accessibility of gametes and the possible control of fertilization parameters, such as time, under *in vitro* conditions should permit studies of the role of the plant cytoskeleton during fertilization in the near future.

Karyogamy after *in vivo* gametic fusion was first shown using transmission electron microscopy in cotton (Jensen, 1964) and later in other plants, including grasses (Van Went, 1970; Wilms, 1981; Mogensen, 1982; You and Jensen, 1985; Hause and Schröder, 1987; Guo-Wei and Jia-Heng, 1992). Three main steps were described and are as follows: (1) the external nuclear membranes become closely apposed or in contact via the endoplasmic reticulum and then fuse; (2) the inner nuclear membranes fuse and bridges are formed between nuclei; and (3) the bridges enlarge and can entrap some cytoplasm. The male chromatin then becomes more diffuse. We have not been able to observe the second step, which probably occurs very rapidly, in the *in vitro* gametic fusion system. However, as under *in vivo* fertilization conditions, we observed the apposition of the external nuclear membranes (Figures 2D and 2E). Later, we observed a remnant of the nuclear envelope (Figure 4B) and noticed that the male chromatin becomes less condensed, as it does under *in vivo* fertilization conditions. The male chromatin volume estimate was higher than before the fusion of nuclei: 134 μm^3 compared to 55 μm^3 before nuclear fusion. Nuclei within isolated sperm cells range in volume from 35.6 to 88.5 μm^3 (Mogensen et al., 1990). After these three main steps, the nucleus of some samples contained two nucleoli, as is probably the case transiently under *in vivo* fertilization conditions in some grasses, such as wheat, maize, and barley (see Batygina, 1974; Mogensen, 1982).

Thus, gametic fusion products generated *in vitro* behaved like zygotes generated *in vivo* with regard to the ultrastructure of their karyogamy. Fusion of nuclei occurred before the zygotic division, as is the case under *in vivo* conditions in grasses and in some animals, including sea urchin (see Longo, 1973). Thus, fusion was premitotic rather than postmitotic, in which nuclei remain distinct until after the first mitotic division (see Longo, 1973; Hu and Zhu, 1979). In addition, *in vitro* karyogamy seems to be similar to nuclear fusion in *in vitro* somatic protoplast fusion systems when studied with transmission electron microscopy (Fowke et al., 1977).

Two important questions about nuclear fusion remain unsolved. First, when does DNA replication take place? In a nonflowering plant, *Ephedra*, which exhibits a double fertilization process (Friedman, 1990), DNA measurements demonstrated that male DNA replication occurs when the male and female nuclei are apposed just before karyogamy (Friedman, 1991). In flowering plants, few data are available. It was demonstrated by flow-cytometric DNA measurement from maize pollen grains that sperm cells are in the G1 phase (Bino et al., 1990). These results are not sufficient to determine the DNA replication time in sperm cell nuclei. However, according to Woodard (1956), sperm and egg nuclei can fuse in the G1 phase in *Tradescantia*.

These results have to be confirmed, at least in maize. The *in vitro* fertilization system could be used to answer this question by obtaining DNA measurements before and after karyogamy, according to the timetable we have established. Second, is there nuclear recognition? Hexaploid wheat \times maize crosses lead to a very efficient karyogamy in wheat egg cells, according to Laurie and Bennett (1990). However, it is not possible to determine from these results whether a nuclear recognition involving conserved signals in maize and wheat takes place or not. Interspecific crosses *in vitro* should allow the analysis of interspecific barriers.

Fate of the Male Cytoplasmic Organelles

What is the fate of mitochondria seen in sperm cells within pollen grains (McConchie et al., 1987) or after isolation (Wagner et al., 1989b)? Because no external cytoplasmic bodies were observed after electrofusion, there is apparently no exclusion of sperm cytoplasm during gametic fusion as occurs *in vivo* in barley (Mogensen, 1988) and probably in cotton (Jensen and Fisher, 1968). Thus, mitochondria seem to be transmitted under our experimental system; however, it is then hard to follow their fate because male and female mitochondria are difficult to distinguish from each other. Some mitochondria with highly contrasted cristae, as seen in isolated maize sperm cells (Wagner et al., 1989b), were observed in some samples (Figure 2E). However, even if these mitochondria originated from the male side, they could have degenerated later (see Mogensen, 1988). Therefore, genetic studies are needed with crosses of different lines or species to determine whether inheritance is uniparental or biparental, as is the case in some animals (Gyllensten et al., 1991; Zouros et al., 1992). It seems that *in vivo* maize mitochondrial inheritance is strictly maternal, according to genetic analysis (Levings and Pring, 1976; Conde et al., 1979).

On the other hand, plastid inheritance should be strictly maternal, as it is under *in vivo* fertilization conditions (for review, see Hagemann and Schröder, 1989), because sperm cells have not been seen to contain plastids either within pollen grains (McConchie et al., 1987; Rusche and Mogensen, 1988) or after isolation (Wagner et al., 1989b; Mogensen et al., 1990).

Conclusion

Our results demonstrate the occurrence of karyogamy in the unique *in vitro* fertilization system performed in flowering plants. As discussed above, the ultrastructure and the time course of karyogamy seem to be similar to *in vivo* fertilization. In addition, the overall ultrastructure of the fusion products compared with isolated egg cell protoplasts (Faure et al., 1992) does not seem to be altered, and the fusion of both gametes probably occurs by fusion of their plasma membranes, as is probably the case *in vivo* (see Russell, 1992). Thus, the cytological data we obtained, together with embryogenesis and regeneration

of fertile plants that are now possible (Kranz and Lörz, 1993), indicate that this gametic fusion system is very promising. Adhesion and cell-to-cell recognition cannot be studied because the gametic fusion is forced. However, such a system should allow studies of early events of fertilization, such as nuclear migration or DNA replication, and embryogenesis. It should also be useful for genetic manipulations.

METHODS

Plant Material, Isolation of Gametes, and Electrofusion of Gametes

The plant material and the methods used for gamete isolation and electrofusion were the same as described by Kranz and Lörz (1993).

Chemical Fixation

Electrofusion products of isolated sperm and egg cell protoplasts were washed in two agarose droplets of $\sim 2 \mu\text{L}$ (1% [w/v] agarose type IX [Sigma], in 0.4 M mannitol, ultralow-gelling temperature) and then introduced by means of a hydraulic system into 5- μL agarose droplets deposited on plastic pieces measuring 0.5 \times 0.5 cm. The agarose was solidified rapidly by cooling the samples for 5 to 10 min at 4°C. These steps allowed the individual cells to be rapidly carried at precise times after electric pulses in fixative (3% [w/v] glutaraldehyde in sodium cacodylic acid buffer) and postfixative (1% [w/v] osmium tetroxide in the same buffer) solutions, as described by Faure et al. (1992). The fixed electrofusion products inside agarose droplets were then dehydrated in a graded ethanol series (25, 50, 70, 95, and 100% [v/v]), infiltrated by Spurr's resin (Spurr, 1969), removed from plastic pieces, and embedded in the same resin polymerized at 70°C for 24 hr.

Microscopy

Twenty-three samples were serially semithin sectioned (3 μm) with glass knives on a Reichert Ultracut E microtome at the Centre de Microscopie Appliquée à la Biologie et la Géologie, Université Lyon I. The sections were observed with a microscope equipped with phase contrast objectives (Laborlux S; Leitz, Wetzlar, Germany). Pertinent sections were photographed and reembedded at the surface of an epoxy block (see Mogensen, 1971). These reembedded sections were serially ultrathin sectioned with a Microstar diamond knife on a Reichert Ultracut E microtome at the Biology Department of Northern Arizona University. Sections were collected on formvar-coated and carbon-stabilized single slot grids and stained with uranyl acetate for 30 min at 40°C and lead citrate for 30 min at 20°C in an LKB Ultrastainer (LKB, Bromma, Sweden). Stained sections were observed at the Biology Department of Northern Arizona University under a JEOL 1200 EX II electron microscope (JEOL LTD., Tokyo, Japan) at 60 kV.

Three-Dimensional Reconstructions

Three-dimensional reconstructions were made according to the methods of Mogensen et al. (1990) and Faure et al. (1992). Serial

photographic prints were prepared from serial ultrathin sections. Tracings of the nuclei were then prepared on plastic overlays and digitized with a digitizing tablet (Summasketch; Summagraphics Corp., Fairfield, CT) and an IBM PC-AT computer. Three-dimensional reconstructions were obtained on a color monitor with the computer program written by Young et al. (1987), and volumes were estimated using a program written by M. Rusche (unpublished results).

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