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Gametes and Fertilization: Maize as a Model System for Experimental Embryogenesis in Flowering Plants

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INTRODUCTION

The involvement of each of the two sperm cells from a pollen tube in separate fusion events characterizes double fertilization: a process that is illustrated in Figure 1. This process, which initiates the development of both the embryo (via the fusion of sperm and egg) and the endosperm (via the fusion of sperm and central cell), was discovered in angiosperms nearly a century ago (Nawaschin, 1898; Guignard, 1899; reviewed in Russell, 1992).

The application of the transmission electron microscope to the study of flowering plant embryogenesis in the early 1960s by Jensen and coworkers (reviewed in Jensen, 1973, 1974) marked the beginning of great strides that have been made in our understanding of embryogenesis since Maheshwari's (1950) comprehensive review. Jensen and Fisher (1968, 1970) confirmed the cellular nature of sperm cells and noted that sperm cells remain connected after their formation from the generative cell division. Jensen (1973, 1974) also showed that the two synergids that typically flank the egg are active, functional cells that play a significant role in the fertilization process. More recent studies, also employing transmission electron microscopy but extending its use to the creation of three-dimensional, computer-generated reconstructions, have led to the concept of the male germ unit, which views the sperm cells and vegetative nucleus as a functional assemblage required for normal gamete transport, recognition, and fusion (see below; Dumas et al., 1984; reviewed in Mogensen, 1992). Because the sperms of a pair are dimorphic in some species, it also became apparent that double fertilization may not be a random event, i.e., that each sperm may be preprogrammed for fusion with either the egg or the central cell (Russell, 1985).

Although the process of double fertilization offers unique opportunities for studying gamete interactions and control mechanisms, it also presents special problems not encountered in animals and nonangiospermous plants. A major deterrent to physiological, molecular, and experimental studies of embryogenesis in angiosperms has been the inaccessibility of the gametes. During the past few years, however, it has become possible in some angiosperm species to isolate male and female sex cells in large enough quantities to allow for the initial phases of such analyses.

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Although sperm cell isolation has been accomplished in several species (for reviews, see Russell, 1991; Theunis et al., 1991; Chaboud and Perez, 1992), female gamete isolation has been less successful in most of these species. In maize, it is possible to isolate large numbers of both gamete types; for this reason and others, enumerated below and in Table 1, maize is emerging as a model system for the study of gametes, fertilization, and experimental embryogenesis. Thus, we have focused this review primarily on recent studies in maize, with reference to other systems where appropriate.

SUITABILITY OF MAIZE FOR THE STUDY OF GAMETES, FERTILIZATION, AND EXPERIMENTAL EMBRYOGENESIS

As shown in Figure 2, the unisexual flowers of maize occur separately in the form of the tassel (staminate flowers; Figure 2A) at the tip of the plant and one or more ears (pistillate flowers; Figure 2F) in the axils of leaves near the middle of the stem. Several million pollen grains are released from the tassel, and 400 to 500 ovules are available per ear. Controlled pollination is much easier in maize than in other cereals, in which emasculation of each flower is required before it can be used as a female parent.

In addition to these useful sexual traits, considerable data are available on the genetics and cytogenetics of maize (Sheridan, 1982). More recently, much attention has been focused on molecular approaches to the study of maize development, including the use of restriction fragment length polymorphism markers (more than 1200 are known today) and on the analysis of some interesting genes and their protein products (Sheridan, 1988; Vollbrecht et al., 1991; Bellmann and Werr, 1992; Veit et al., 1993, this issue).

Recent Progress in Gamete Isolation and Manipulation

The course of contemporary research in sperm cell biology was plotted at the first sperm cell biology conference, held in Flagstaff, Arizona, in 1985. This conference led to the

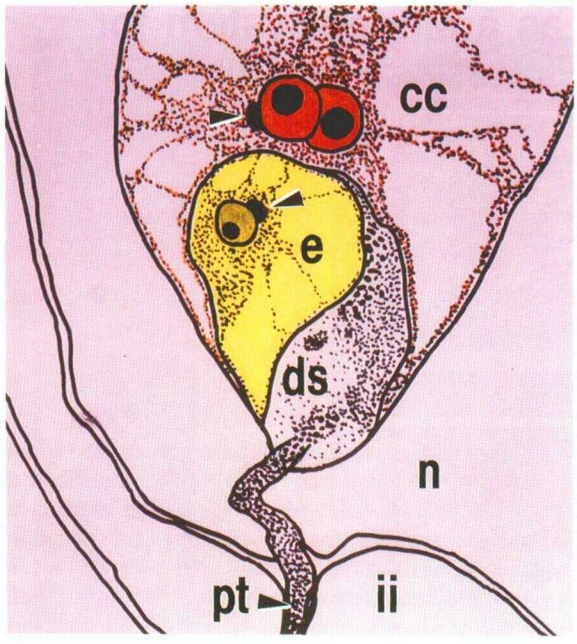

Figure 1. Double Fertilization in Maize.

The pollen tube (pt) has entered the degenerated synergid (ds) and discharged through a terminal pore. The two sperm cells have traversed the synergid and their nuclei (unlabeled arrowheads) are seen fusing with the egg (e) nucleus (yellow) and the polar nuclei (red) within the central cell (cc). ii, inner integument; n, nucellus. Adapted with permission from Van Lammeren (1986) according to R. Mol and C. Dumas' unpublished results on genotype A 188.

development of several procedures for isolating en masse viable sperm cells of maize, as depicted in Figures 2A to 2E (Dupuis et al., 1987; Yang and Zhou, 1989) and for characterizing them (Russell et al., 1990; Roeckel et al., 1991), as shown in Table 2. Table 3 shows that a parallel approach has been developed to successfully isolate living embryo sacs and egg cells for further manipulation and characterization (Wagner et al., 1989b; Figures 2F to 2J). Several recent reviews (Roeckel et al., 1991; Theunis et al., 1991; Chaboud et al., 1992; Russell, 1993, this issue) have addressed this topic and its influence on new developments in the study of fertilization in angiosperms (see also reviews in Russell and Dumas, 1992).

Procedures for the Regeneration of Fertile Plants from "Artificial" and "Natural" Zygotes

Recently developed techniques for the isolation and manipulation of male and female gametes offer a new way to study fertilization through the creation of zygotes induced by electrofusion, as illustrated in Figures 3A and 3B (Kranz et al., 1991a, 1991b). In addition, after several years of trials, a critical step has been reached with the possibility of regenerating fertile plants from embryo sacs isolated at the zygote stage (Campenot et al., 1992; Mol et al., 1993). This progress in gamete technology and in the isolation and regeneration of both "artificial" and "natural" zygotes provides a promising approach for genetic transformation of maize, which is difficult to obtain by other methods (e.g., Gordon-Kamm et al., 1990; D'Halluin et al., 1992). In addition, such zygotes are very powerful tools for the molecular analysis of fertilization and the earliest steps of embryogenesis.

FROM POLLEN TO GAMETE ISOLATION

Pollen Organization and the Male Germ Unit

The pollen grain is a unique multicellular organism. In most angiosperms, it consists of a generative cell (sperm mother cell) surrounded by the vegetative cell (future pollen tube); thus, it is termed "bicellular" pollen. Maize pollen is termed "tricellular," because at pollen maturity the generative cell has already divided to form two sperm cells (Bedinger, 1992; Figure 2C).

Many studies conducted over the past decade have documented that the sperm cells either remain connected or become reunited after generative cell division and that one or both sperms forms a close association with the vegetative nucleus. Because the nuclear and cytoplasmic DNA–containing compartments of male heredity are physically linked, they have been termed the male germ unit (Dumas et al., 1984; Mogensen, 1992). Clear evidence of the validity of this concept has been provided by the isolation of the intact male germ unit (Matthys-Rochon et al., 1987). The male germ unit is established before or shortly after pollination, and its components typically maintain their association up to the time of pollen tube discharge into one of the two synergids that flank the egg cell (Russell, 1992; Figure 1). Thus, the male germ unit appears to function as a vehicle for gamete transmission, recognition, and fusion during double fertilization (see reviews in Mogensen, 1992; Russell, 1992, 1993, this issue). However, the male germ unit is usually disrupted during the process of sperm cell isolation (with a notable exception in *Brassica*; see Matthys-Rochon et al., 1987), and the sperm cells are found singly in the isolation medium. This special association of three different haploid cells may result from the expression of several sets of pollen-specific genes (Mascarenhas, 1990; McCormick, 1991) and from interactions between anther-specific gene products and the innermost layer of the anther, the tapetum (Koltunow et al., 1990).

Because in some plants, the two sperms of a pair differ greatly from one another in size, shape, and organellar content, it was suggested that they may be preprogrammed to fuse with either the egg or the central cell. Such preferential fertilization has been demonstrated to occur in *Plumbago*, in which the smaller, plastid-rich sperm almost always fertilizes the egg cell (Russell, 1985; also, see Russell, 1993, this issue). The sperms of a pair in maize do not differ greatly from each other

morphologically (McConchie et al., 1987; Rusche, 1988; Rusche and Mogensen, 1988); nevertheless, directed fertilization is known to occur, at least in lines containing B-chromosomes. In this system, there is a nondisjunction of the B-chromatids during generative cell division, resulting in only one sperm receiving the B-chromosomes. This sperm most often fuses with the egg cell (Roman, 1948; Chaboud et al., 1992).

Pollen Quality: A Prerequisite to Gamete Isolation

Depending on the genotype, a healthy tassel generally sheds several million pollen grains during a 1-week period. Pollen shedding usually begins 1 or 2 days before silks appear on the ear shoots.

The pollen grain is virtually a dehydrated organism, and its "quality" has been defined as its ability to fertilize and produce

Table 1. Maize: Model System for Cellular and Molecular Analysis of Gametes, Fertilization, and Early Embryogenesis

Interesting Traits	Characterization	References
Pollen		
Pollen collection	Several million/inflorescence	Sheridan (1982)
Long-term pollen storage (LN_2)	Possible in many genotypes	Barnabas (1985)
Pollen from in vitro–cultured tassel	Available	Parredy et al. (1989)
In vitro pollen maturation from microspore	Difficult	Dupuis and Pace (1992)
In vitro pollen tube growth	Available	Pfahler (1967)
In vitro pollen tube growth velocity	5 to 8 mm/hr	Dupuis and Dumas (1989)
Ovule number per spike	400 to 500	Sheridan (1982)
Ovule and silk receptivity	Assessed by silk length	Dupuis and Dumas (1989)
In vitro ovule/pollination/regeneration	Available	Dupuis (1992)
Pollination/Fertilization		
Single pollen grain pollination	Available	Kranz and Lörz (1990)
Embryo sac isolation	Available	Wagner et al. (1989b)
Egg cell isolation	Difficult	Wagner et al. (1989b)
Central cell isolation	Difficult	Wagner et al. (1989b)
"Artificial zygote" formation	Electrofusion	Kranz et al. (1991a, 1991b)
"Artificial zygote" regeneration	Available	Kranz and Lörz (1993)
Fertilized embryo sac regeneration	Available	Campenot et al. (1992); Mol et al. (1993)
Nonzygotic embryogenesis		
Somatic embryogenesis	Difficult; genotype dependent	Freeling and Walbot (1993)
Androgenic embryogenesis	Difficult; genotype dependent	Freeling and Walbot (1993)
Gynogenetic embryogenesis	Difficult; genotype dependent	Freeling and Walbot (1993)
Regeneration/Transformation		
Microinjection in microspore	Available	Gaillard et al. (1992)
Microinjection in pollen	Available	Kranz and Lörz (1990)
Microinjection in embryo sac	Available	E. Matthys-Rochon (unpublished results)
Transformation	Established, but difficult	Gordon-Kamm et al. (1990); D'Halluin et al. (1992)
Regeneration from protoplasts	Difficult; genotype dependent	Rhodes et al. (1988)
Other various traits		
Insertional mutagenesis	Available	Freeling and Walbot (1993)
Embryo mutants	Numerous	Clark and Sheridan (1991); Meinke (1991); Walbot (1991)
Long-term storage of embryos	Available	Delvallée et al. (1989)
Endosperm mutants	Numerous	Clark and Sheridan (1991); Meinke (1991); Walbot (1991); Freeling and Walbot (1993)
B–A translocations	Available	Sheridan (1982); Freeling and Walbot (1993)

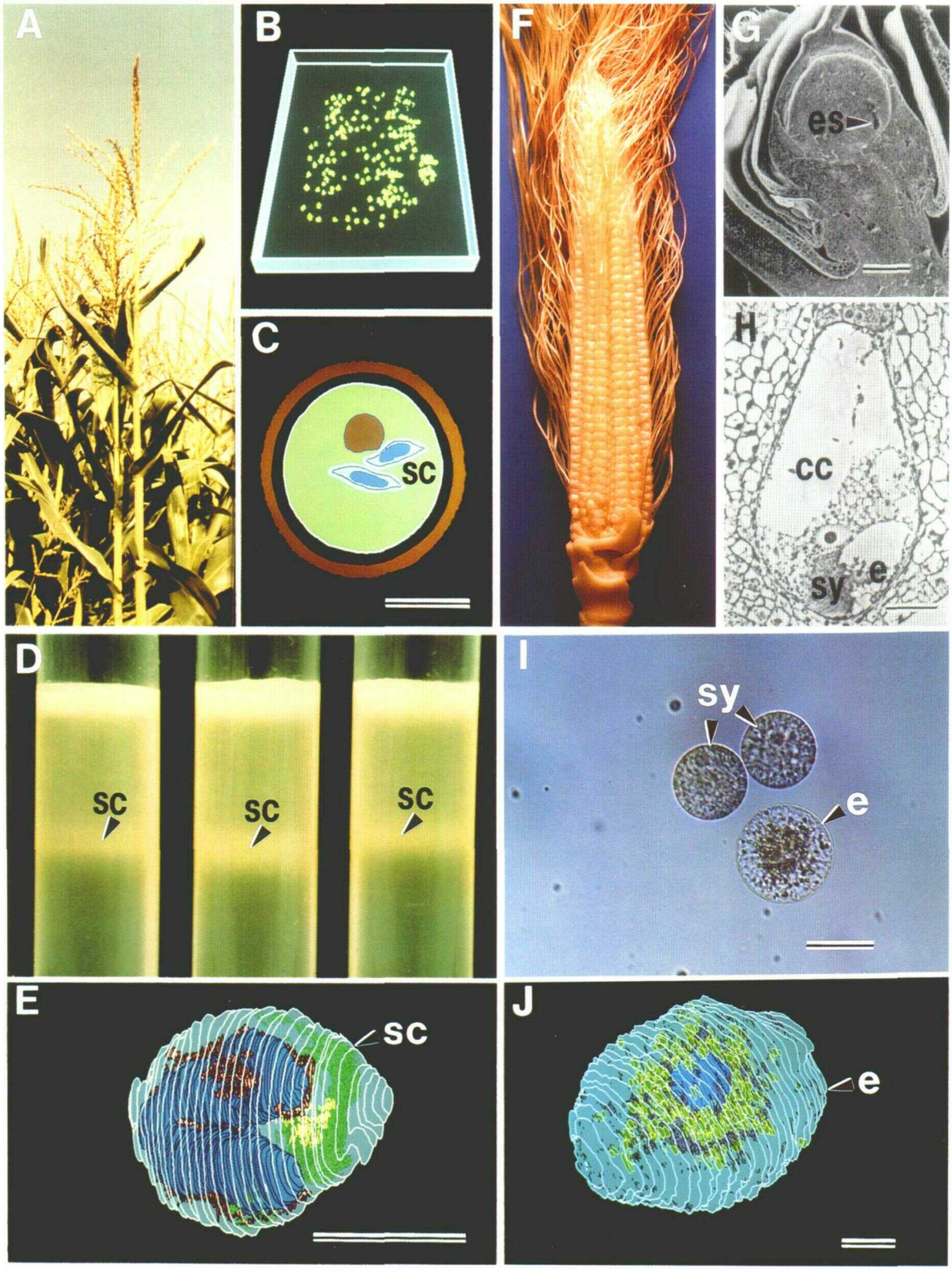

Figure 2. Steps in the Isolation of Male and Female Gametes of Maize.

Table 2. Maize Sperm Cells: Technical Characteristics for Cellular and Molecular Analysis

Feature	Characterization	References
Pollen diameter	100 μm	M. Rusche (personal communication)
Sperm cell (SC) diameter	5 to 7 μm	Cass and Fabi (1988); Wagner et al. (1989a); Mogensen et al. (1990)
SC volume	163 to 253 μm^3	Wagner et al. (1989a); Mogensen et al. (1990)
SC volume/pollen volume	0.002% to 0.2%	Mogensen et al. (1990)
Volume of total SC organelles[a]/SC volume	35.78	Mogensen et al. (1990)
SC volume/pollen plasma membrane volume	0.5% to 2%	Mogensen et al. (1990)
Volume of SC nucleus/SC volume	32.34	Mogensen et al. (1990)
Volume of SC plastids	0	Mogensen et al. (1990)
3-dimensional reconstruction and characterization of SC	Available	Mogensen et al. (1990)
"En masse" SC isolation	10^6 to 10^7; 80 to 90%[b]	Dupuis et al. (1987); Zhang et al. (1992b)
SC-enriched fraction	Available	Chaboud and Perez (1992); Chaboud et al. (1992)
SC survival	72 hr at 20°C	Zhang et al. (1992a)
Long-term SC storage medium	Available	Chaboud and Perez (1992); Chaboud et al. (1992)
SC analysis by cell sorter	Available	Zhang et al. (1992b)
SC isolation procedure from pollen tube	Not available	
SC cDNA library	Available	A. Chaboud and C. Breton (unpublished results)
Isolation of SC from B-chromosome line	Available	A. Chaboud, H. L. Mogensen, and C. Dumas (unpublished results)
SC cytology in vivo	Documented	McConchie et al. (1987); Rusche (1988); Rusche and Mogensen (1988)

[a] Excluding vacuoles.
[b] Total number of isolated SC obtained from a single isolation procedure; percentage of isolated cells recovered.

seeds. However, the water content of living pollen grains varies considerably among different families, with most values lying between 15 and 35% of the fresh weight at anthesis, with some extremes of 8% water in poplar pollen and 60% water in maize pollen. Although it is easy to evaluate pollen viability, pollen quality is much more difficult to assess by routine tests. In maize, pollen longevity is very short under natural conditions, from 20 min to 2 hr. The most rapid method available to determine pollen viability is a cytological technique, the fluorochromatic reaction (FCR) (Heslop-Harrison and Heslop-Harrison, 1970), which indicates the structural integrity of the plasma membrane.

Figure 2. (continued).

(A) to **(E)** Isolation of male gametes.
(A) Tassels containing staminate flowers.
(B) Collection of pollen on an aluminum sheet.
(C) Schematic of a maize pollen grain containing two sperm cells. Bar = 40 μm.
(D) Centrifuge tubes with purified sperm cell fraction in Percoll gradient.
(E) Computer-assisted reconstruction of an isolated sperm cell based on serial ultrathin sections. Reproduced with permission from Mogensen et al. (1990). Bar = 5 μm.
(F) to **(J)** Isolation of female gametes.
(F) Young ear of maize with pistillate flowers.
(G) Longisection of a floret showing the position of the embryo sac within the ovule. Reproduced with permission from Wagner et al. (1990). Bar = 500 μm.
(H) Longisection of the embryo sac showing the egg, central cell, and synergid. Reproduced with permission from Wagner et al. (1990). Bar = 50 μm.
(I) Isolated egg cell and two synergids. Bar = 50 μm.
(J) Computer-assisted reconstruction of an isolated egg cell based on serial ultrathin sections. Reproduced with permission from Faure et al. (1992). Bar = 10 μm.
sc, sperm cells; es, embryo sac; e, egg; cc, central cell; sy, synergid.

Table 3. Isolation and Characterization of the Embryo Sac and Female Gametes

Type of Cell	Data	References
Embryo sac		
Embryo sac isolation	Available	Wagner et al. (1989b)
Cytology of embryo sac	Available	Diboll and Larsen (1966); Diboll (1968); Russell (1979); Van Lammeren (1986)
Embryo sac localization, 3-dimensional reconstruction	Available	Wagner et al. (1990)
Embryo sac microinjection	Available	E. Matthys-Rochon (unpublished results)
Embryo sac cDNA library	Not yet available	
Egg cell		
Egg cell isolation	Difficult	Wagner et al. (1989b)
Isolated egg cell (3-dimensional reconstruction)	Available	Faure et al. (1992)
Egg cell longevity	Two weeks at 24°C	Kranz et al. (1991a)
Egg cell volume/sperm cell volume	300	Faure et al. (1992)
Volume of egg nucleus/egg volume	1.71%	Faure et al. (1992)
Volume of total egg organelles[a]/egg volume	8.98%	Faure et al. (1992)
Volume of egg plastids/egg volume	2.95	Faure et al. (1992)
Egg cell cDNA library	Not yet available	
Synthesis and accumulation of ribosomes	Studied	Dow and Mascarenhas (1991a, 1991b)
Central cell		
Central cell isolation	Difficult	Wagner et al. (1989b)
Isolated central cell (3-dimensional reconstruction)	Not yet available	
Central cell cDNA library	Not yet available	
Synthesis and accumulation of ribosomes	Available	Dow and Mascarenhas (1991a, 1991b)

[a] Vacuoles excluded.

However, a multidisciplinary approach that includes FCR testing, seed set, in vitro germination, freeze fracturing of pollen grains, and a nondestructive method, nuclear magnetic resonance, is necessary to follow pollen quality effectively during aging. To date, such an approach has been used only with maize pollen. A strict correlation has been found between maize pollen water content and plasma membrane integrity and pollen viability. During pollen aging, a natural dehydration occurs that induces major modifications in the plasma membrane, and beyond a critical point, there is a dramatic modification of the water content, as determined by characteristic values of proton mobility. At this point, the pollen loses its quality (Kerhoas et al., 1987). These data partially explain why pollen is especially sensitive to environmental stresses such as drying and temperature (Sheridan, 1982), and why it is so difficult to store pollen in liquid nitrogen (Barnabas, 1985; C. Digonnet-Kerhoas, G. Gay, and C. Dumas, unpublished results).

There is little hope for fertilization if the temperature rises much above 36°C. The synthesis of heat shock proteins (HSPs) has been analyzed in male and female tissues to establish a relationship between physiological and molecular responses to heat shock. Under heat shock conditions (up to 36°C), the synthesis of a typical set of HSPs is induced in the female tissues. HSPs are also normally inducible during microsporogenesis but not in mature pollen, which at these temperatures is unable to effect fertilization (Dupuis and Dumas, 1990a).

Approaches have been developed to study pollen formation under controlled conditions, such as the in vitro culture of maize tassels, a technique that has been used successfully in several genotypes (Parredy and Greyson, 1985; Parredy et al., 1989). Another approach, which, so far, has had limited success, involves maturation of pollen in vitro from cultured microspores (Dupuis and Pace, 1992).

Sperm Cell Isolation Procedures

Isolated sperm cells can easily be obtained in large numbers from maize pollen (an average of 10^6 to 10^7 cells are obtained per isolation procedure) by osmotic shock coupled with a pH shock (Dupuis et al., 1987). The sperm cells are then separated from pollen contaminants by filtration and discontinuous Percoll gradient centrifugation (Figures 2B to 2E). After isolation, sperm cell viability is generally evaluated with the aid of the FCR. An improved isolation procedure has recently been published (Roeckel et al., 1991). In addition, recent attempts have been made to develop a long-term sperm cell storage medium for further molecular analysis (Roeckel et al., 1991; Zhang et al., 1992a, 1992b).

Physiological Characterization of Sperm Cells

Electron microscopy has demonstrated that isolated sperm cells of maize are true protoplasts, i.e., intact cells without a cell wall (Dupuis et al., 1987; Cass and Fabi, 1988; Wagner et al., 1989a). This has recently been confirmed by preliminary

patch-clamp measurements (A. Chaboud, M. de Barros Lopes, C. Breton, R. Pidulsky, O. Rougier, and C. Dumas, unpublished results). Other studies have shown that the presence of ATP (a nucleotide known to be an indicator of life and used to test the fertility of human sperm) is a good indicator of the metabolic potential of these cells (Roeckel et al., 1991; Chaboud and Perez, 1992). In addition, ^{35}S-methionine labeling experiments clearly show that these isolated cells are able to synthesize new proteins (P. Roeckel and C. Dumas, unpublished results).

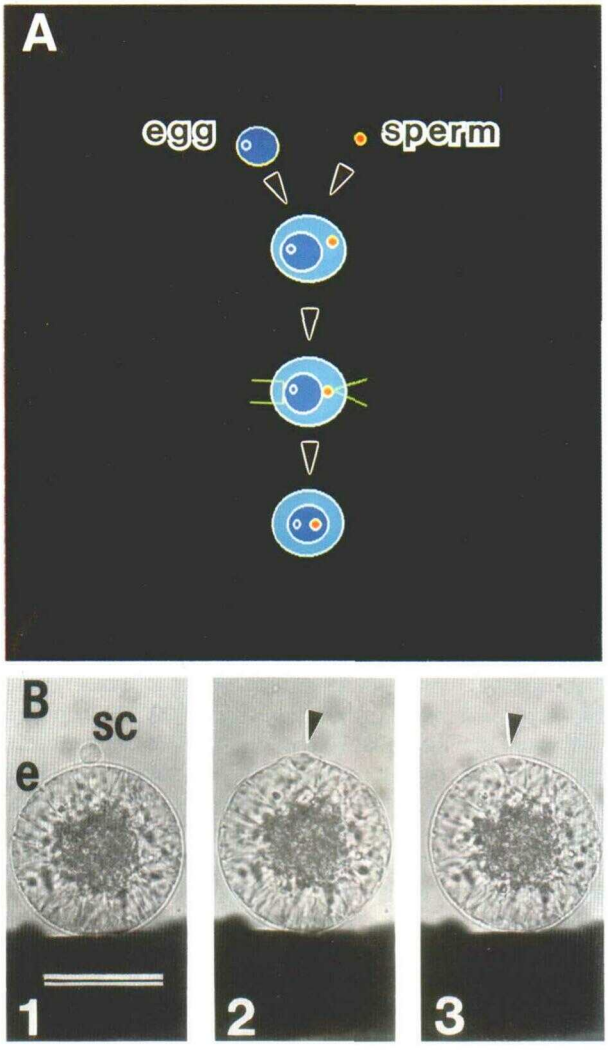

Figure 3. In Vitro Fertilization in Maize Facilitated by Electrofusion.

(A) Isolated sperm and egg cells are placed into a fusion droplet (light blue), dielectrophoretically aligned with electrodes (green), and electrically fused (adapted with permission from Kranz et al., 1991a).
(B) Sequence of electrofusion between isolated egg (e) and sperm (sc) cells. Arrowheads show entrance site of the sperm cell. The time interval, after the DC pulse, from 1 to 3 was less than one second. Reproduced with permission from Kranz and Lörz (1991). Bar = 50 μm.

The inner plasma membrane of the vegetative cell has been shown to surround the sperm cell plasma membrane by both conventional transmission electron microscopy (McConchie et al., 1987; Rusche, 1988; Rusche and Mogensen, 1988) and the freeze–fracture method (Southworth, 1992). During in vitro isolation, this vegetative inner membrane is lost, a phenomenon that also occurs during pollen tube discharge within a synergid prior to fertilization. Such a phenomenon has been compared to capacitation in animal gametes, which prepares sperm cells to fuse with the female gametes (Dumas et al., 1984; Russell, 1993, this issue).

Gamete Dimorphism

Success in isolating intact, viable, and functional maize gametes offers the possibility of identifying the specific determinants involved in fertilization, which are thought to be borne on the interacting gamete plasma membranes (Dumas et al., 1984; Chaboud et al., 1992). Such studies may uncover differences in the membrane proteins of dimorphic sperm cells. The construction of a monoclonal antibody library directed against the surface of viable sperm cells has been designed (Hough et al., 1986) and developed in maize as well as in *Brassica campestris* and *Plumbago zeylanica* (reviewed in Chaboud and Perez, 1992). However, a common problem is that the strongly reactive lines are directed to debris or cell contaminants rather than to plasma membrane surface determinants. These studies clearly indicate the necessity of obtaining more highly purified gamete preparations, free from pollen cytoplasmic contaminants (Chaboud et al., 1992). However, if the purity of the fraction is increased significantly, the number of pure living sperm cells or amount of pure plasma membrane fraction decreases dramatically (see Table 2). In parallel, in vitro fertilization of isolated gametes without the aid of electrofusion or polyethylene glycol has recently been developed (J.-E. Faure, C. Digonnet-Kerhoas, and C. Dumas, unpublished results). This system will allow selection of fusion-inhibiting monoclonal antibodies and, thus, the isolation of putative candidate molecules involved in gamete recognition (A. Chaboud and C. Dumas, unpublished results).

Nuclear dimorphism in maize sperms, in the form of B-chromosomes usually being present in only one of the sperms (Roman, 1948), is a potentially useful trait for separating one type of sperm from the other. However, preliminary trials using flow cytometry suggest that detection of DNA differences between isolated sperms would require ~10 B-chromosomes (H. L. Mogensen and A. Chaboud, unpublished results). Unfortunately, when the number of B-chromosomes is this high, their distribution to the sperms becomes random (Carlson, 1988). There are small structural differences between the sperm pair of maize even in the absence of B-chromosomes (McConchie et al., 1987; Rusche, 1988; Rusche and Mogensen, 1988), but these differences do not allow the detection of two sperm types within a population of isolated sperm cells (Wagner et al., 1989a; Mogensen et al., 1990).

FROM OVULE TO FEMALE GAMETE ISOLATION

In angiosperms, the embryo sac represents the female gametophytic generation (see Reiser and Fischer, 1993, this issue). This megagametophyte is deeply embedded in the protective sporophytic envelope of the ovary and ovule as a consequence of angiospermy (Figures 2G and 2H). The female germ unit (Dumas et al. 1984; Huang and Russell, 1992) is seen as including the egg apparatus (the egg and synergids) and the central cell because in most species, all of these components are involved in the fertilization process, which includes pollen tube attraction to the ovule and one synergid, pollen tube discharge, sperm cell transport, and gamete fusion (Figure 1; Russell, 1992). The special environment within the penetrated synergid, which typically degenerates before pollen tube arrival, is undoubtedly critical to the normal process of syngamy. For instance, the calcium concentration in the synergids is high. Simulating these conditions will be a major but necessary challenge to achieve fertilization in vitro without the application of electric pulses.

One consequence of the fact that the embryo sac is embedded deep within maternal tissues is that it is difficult or impossible to make physiological measurements and direct morphological observations. Several methods have been developed for isolating embryo sacs from ovular tissues, including partial enzymatic digestion of ovules followed by manual dissection, squashing (Zhou, 1985; Wagner et al., 1989b), or microdissection (Mol et al., 1993). Using these methods, it is possible to obtain viable embryo sacs and, after an additional, very brief enzymatic treatment, living gametes (both egg, Figures 2I and 2J, and central cells) can be separated (Wagner et al., 1989b). The debris is removed by filtration through several nylon filters, and embryo sacs and gametes are then placed in the appropriate survival medium.

Studies using a molecular approach to investigate the components of the maize embryo sac are just getting underway (Reiser and Fischer, 1993, this issue). As part of a project to isolate genes expressed in the female gametophyte, Dow and Mascarenhas (1991a, 1991b) have employed in situ nucleic acid hybridization to determine the relative number of ribosomes within the cells of the embryo sac of maize. In the mature embryo sac, the central cell contains ~200 times as many ribosomes as are present in a nucellar cell, ~7 times as many ribosomes as are contained in the egg cell, 14 times as many ribosomes as in a synergid, and ~80 times as many ribosomes as in each antipodal cell. During embryo sac maturation, the accumulation of ribosomes appears to proceed at a constant and high rate, with the highest rate in the developing central cell.

Mature embryo sacs must be isolated from receptive ovules, that is, ovules at a stage during which pollination will be effective. However, ovule receptivity is a complex and unclear physiological parameter that is still largely ignored and difficult to assess. In maize, female receptivity can be estimated by the total silk length, which corresponds to the maturation state of spikelets (Dupuis and Dumas, 1990b). Polyacrylamide gel electrophoresis of silk proteins shows that ~10 proteins seem to be correlated with the acquisition of silk receptivity (Dupuis, 1992). In addition to these putative protein markers, the level of expression of a gene coding for a serine/threonine protein kinase (Walker and Zang, 1990) seems to be positively correlated with silk receptivity (R. Perez, A. Chaboud, and C. Dumas, unpublished results).

EGG CELL CHARACTERIZATION

The egg cell of maize has been well characterized cytologically at both the light and electron microscope levels (Table 3; Russell, 1993, this issue). Unique features include the presence of highly elongated, branched mitochondria and a large, chalazally positioned vacuole (Diboll and Larson, 1966; Diboll, 1968; Van Lammeren, 1986; Wagner et al., 1990). Upon isolation, the egg protoplast is spherical, with numerous small vacuoles surrounding the centrally located organelles, among which the nucleus is positioned near the periphery (Faure et al., 1992; Figures 2I and 2J). Until it is possible to isolate maize eggs in larger numbers, characterization of these cells at the molecular (e.g., cDNA libraries) and physiological (ATP production, protein synthesis, etc.) levels will be somewhat restricted.

IN VITRO POLLINATION: INTEREST AND LIMITS

The reproductive phase of the plant life cycle is difficult to analyze in the field because of the number of factors involved: among these are female receptivity, pollen quality, and environmental influences. An in vitro pollination system can bypass many of these variables; such an approach, followed by embryo rescue, has generally been used to obtain wide hybridization in higher plants (Zenkteler, 1990, 1992). In vitro pollination in maize (Sladky and Havel, 1976; Gengenbach, 1977) is typically performed by collecting unpollinated ears from the plant; segments of the cob, with two adjacent rows of ovaries attached, are then dissected under sterile conditions. These segments are placed on nutrient agar in a Petri dish, and the silks are pollinated. This technique allows the measurement and experimental manipulation of pollen tube growth within the silk as well as an appreciation of the kinetics of fertilization (Dupuis and Dumas, 1989; Booy et al., 1992). Successful fertilization has been obtained with as few as one pollen grain per silk (Kranz and Lörz, 1990).

The in vitro pollination technique has also been used successfully to separate pollen and ovule behavior (e.g., heat stress tolerance; Dupuis, 1992). This technique can be used to classify different genotypes with regard to stress responses (e.g., to temperature, heavy metal, and osmotic stresses), because in vitro conditions are reproducible. In this regard, Higgins and Petolino (1988) have observed a genotype effect on in vitro pollination success.

FROM NATURAL AND ARTIFICIAL ZYGOTES TO PLANT REGENERATION

A major challenge to plant developmental biologists is the establishment of a successful system for studying the processes of morphogenesis following zygote formation. Such a system is readily available in animals (in vitro fertilization) and in lower plants such as algae (Dale, 1991), but, as outlined in Table 4, until recently no progress had been made in flowering plants. The combination of egg and sperm cell isolation with the technical procedures used for fusing plant protoplasts has opened a new window in gamete technology that was not expected only a few years ago (see Chapman et al., 1985).

Artificial zygotes have been developed by Kranz et al. (1991a, 1991b) from electrofusion of enzymatically or mechanically isolated gametes (Figures 2A and 2B; Table 4). Such zygotes have been shown to be capable of developing into fertile, diploid plants possessing characteristics of both parents (Kranz and Lörz, 1993). The kinetics of karyogamy in such zygotes have been analyzed by classic light and electron microscopy, as well as by reembedding semithin sections and three-dimensional computer-aided reconstruction (Faure et al., 1993; Table 4).

Fusion of isolated gametes without electrofusion has recently been achieved. However, in this case, nuclear fusion must be verified, and plant regeneration needs to be carried out (J.-E. Faure, C. Digonnet-Kerhoas, and C. Dumas, unpublished results).

Another possible method to examine the zygote and its products is to isolate the embryo sac just after fertilization, that is, from 6 to 8 hr after pollination, depending on the genotype and the environmental conditions, particularly temperature. Successful isolation strategies have been developed by two groups (Campenot et al., 1992; Mol et al., 1993; Table 4). Embryo sacs were isolated by enzymatic treatment or microsurgical dissection at the zygote or early embryo stage. The "isolated" zygotes and early embryos were surrounded by the cells of the embryo sac as well as some cells of the nucellus. Using this method, it is possible to regenerate complete plants.

CONCLUSIONS

Although double fertilization was first described by Nawaschin in 1898 and a considerable amount of work has been done on this unique feature of flowering plants, including the study of its possible evolutionary origin (Friedman, 1992), very little progress has been made in terms of molecular analysis. New opportunities are now being provided by in vitro gamete isolation procedures (Dumas and Russell, 1992) and by the development of methods for regenerating electrofused zygotes (Kranz and Lörz, 1993), for injecting sperm cells or sperm nuclei into the isolated embryo sac to mimic fertilization (E. Matthys-Rochon, R. Mol, P. Heizmann, and C. Dumas, unpublished results), and for regenerating in vitro zygotes or embryos

Table 4. Artificial and Natural Zygotes and Zygotic Embryogenesis in Maize

Type of Zygote	Property	References
"Artificial zygote"		
"Artificial zygote" by electrofusion	Available	Kranz et al. (1991a, 1991b)
Karyogamy in electrofused zygote	Demonstrated	Faure et al. (1993)
cDNA library of "artificial zygote"	Not yet available	
Fertile plant regeneration from electrofused zygote	Available	Kranz and Lörz (1993)
"Artificial zygote" without electrofusion	Available	J.-E. Faure, C. Digonnet-Kerhoas, and C. Dumas (unpublished results)
"Natural zygote"		
From isolated fertilized embryo sac	Available	Mol et al. (1993)
Fertile plant regeneration from isolated fertilized embryo sacs	Available	Campenot et al. (1992); Mol et al. (1993)
Microinjection into isolated embryo sacs	Available	E. Matthys-Rochon (unpublished results)
Fertile plant regeneration from two-celled proembryos[a]	Available	Campenot et al. (1992); Mol et al. (1993)
Fertile plant regeneration from isolated embryos (2 DAP)[b]	Available	Campenot et al. (1992); Mol et al. (1993)
cDNA library of isolated embryos (5 DAP)[b]	Available	C. Breton (unpublished results)

[a] From isolated embryo sacs.

[b] Following natural pollination and fertilization; DAP, days after pollination.

isolated from fertilized embryo sacs (Campenot et al., 1992; Mol et al., 1993). cDNA libraries of gametes and early stages of zygotic development (A. Chaboud, M. de Barros Lopes, C. Breton, R. Pidulsky, O. Rougier, E. Matthys-Rochon, and C. Dumas, unpublished results) may provide new tools for the molecular analysis of fertilization and early embryo development.

It may soon be possible to develop genetically transformed maize by microinjecting transformed sperm into the embryo sac rather than by using transformed pollen grains (Roeckel et al., 1992). The latter procedure has failed because of the presence of very active nucleases within the pollen wall and in the stigma that are able to destroy a foreign plasmid within 1 min (Roeckel et al., 1988).

Isolated sperm, egg, and central cells, as well as zygotes, offer new possibilities for studying the cellular and molecular physiology of fertilization (Dumas and Russell, 1992; Kranz et al., 1992). The recent development of in vitro fertilization methods that do not require electrofusion (J.-E. Faure, C. Digonnet-Kerhoas, and C. Dumas, unpublished results) may provide a useful assay for elucidating the barriers that prevent polyspermy in plants and the factors that control cell–cell adhesion and fusion. Finally, access to the earliest steps of fertilization and embryogenesis makes their molecular analysis possible, an eventuality that seemed improbable only a few years ago (Goldberg et al., 1989). In this way, genes specifically expressed in sperm cells might, for example, be compared with genes specifically expressed in pollen (see Mascarenhas, 1993, this issue; McCormick, 1993, this issue). Control of cell polarity and the first division that leads to two embryo cells (the basal cell, the origin of the suspensor, and the apical cell, the origin of the embryo proper; see West and Harada, 1993, this issue) might be analyzed from cDNA libraries developed from egg cells.

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