homoepitaxial growth of diamond on diamond substrates where it is imperative to suppress secondary nucleation events. The observation of growth without new nucleation suggests that deposition on a single-crystal diamond substrate may produce further single-crystal material.

We have demonstrated that diamond can be grown from $\cdot CH_3$ and H· with an arrangement by which the reactive species are segregated until they impact the growth substrate. Growth occurs at the lower end of temperatures seen in filament-assisted diamond CVD and requires the presence of diamond seed crystals.

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An in Vitro System for Adhesion and Fusion of Maize Gametes

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The development of in vitro fertilization systems in flowering plants is important for understanding and controlling the mechanisms of fertilization. Here a method is described in which isolated maize gametes fuse. In a medium containing 5 mM calcium chloride, sperm and egg cells adhere for several minutes and then fuse within 10 seconds. The method is specific to male-female gamete pairs and results in 80 percent fusion, whereas fusions with other combinations of gametic and mesophyllic cells are less frequent. Eggs fertilized in vitro do not fuse with additional male gametes, which suggests that a block to polyspermy exists.

Studies using in vitro fertilization systems in animals and lower plants have led to a better understanding of the critical first steps of fertilization, including species-specific recognition (*1*), binding and fusion of gametes (*2*), intracellular signaling (*3*), and blocking of polyspermy (*4*, *5*). In contrast, little is known about the process of double fertilization that is characteristic of flowering plants (*6*). During double fertilization, two sperm cells, each without a cell wall, are released from the pollen tube into a cell of the female gametophyte, the synergid. The two sperm cells move through the degenerated synergid, enter the intercellular space between the two adjacent female gametes—the egg cell and the central cell—and fuse with them to form the embryo and a nutritive tissue, the endosperm (*7*). All of these steps occur within the embryo sac embedded in the ovule, which is itself enclosed in the flower ovary. Thus the main barrier to studying fertilization has been the inaccessibility of the female gametes and the zygotes. This has restricted our knowledge of double fertilization largely to microscopic observations. For better understanding of the molecular and cellular events of fertilization, methods that allow the in vitro fusion of gametes are important. Over the past few years, techniques to isolate gametes from the paternal and maternal structures have been described (*8*), and more recently, in vitro fusion of gametes has been achieved in maize by use of electrofusion (*9*). This approach leads to karyogamy (*10*) and fertile plant regeneration (*11*). However, electrofusion does not allow studies of gametic recognition and fusion (*12*), because mesophyll protoplasts as well as sperm cells can be induced to fuse with the eggs (*13*). We have therefore developed a gametic fusion system that more closely resembles the in vivo situation.

We isolated gametes from the inbred maize line A188 (*14*). Male gametes were released from freshly collected pollen grains (*9*, *15*) after a pH shock in 0.5 M mannitol. Egg cell and central cell protoplasts were isolated from the ovules by enzymatic treatment followed by manual microdissection (*9*, *16*, *17*). Pairs of male and female gametes isolated under sterile conditions were then combined in a simple fusion medium composed of 0.5 M mannitol containing 1 mM, 5 mM, or 10 mM calcium chloride ($CaCl_2$) (*18*, *19*). The fusion medium was delivered in droplets of 3 to 4 μl under mineral oil on a glass cover slip with siliconized edges (*9*, *20*). The isolated gamete protoplasts were transferred into the droplets with a manual tube clamp (Micropipex, Assistent 558/E) connected to glass microcapillaries with Teflon tubing (*21*). The male and female gametes were then moved into contact by use of glass microneedles (*21*).

At each of the three $CaCl_2$ concentrations studied, we observed adhesion (*22*) of the sperm cell with the egg cell protoplast, followed by fusion (Fig. 1) (*23*). In 1 mM $CaCl_2$, 15.4% of sperm-egg pairs fused; in 5 mM $CaCl_2$, 79.7% fused; and in 10 mM $CaCl_2$, 66.7% fused. When fusion did not occur, a new sperm cell was introduced in each droplet and placed in contact with the egg. After two new attempts at fusion, 15.4% of the egg cell protoplasts fused with sperm cells in 1 mM $CaCl_2$, 96.9% in 5 mM $CaCl_2$, and 79.2% in 10 mM $CaCl_2$. The lower percentage of fusions in 1 mM $CaCl_2$ suggests that there is a calcium requirement for gamete fusion, as is the case in mammals (*2*, *24*).

The fusion of gametes occurred rapidly. In 5 mM $CaCl_2$, adhesion (*22*) generally lasted 3 min 50 s (±1 min 50 s), after which time most of the fusions occurred in less than 10 s. Within the first 15 min after

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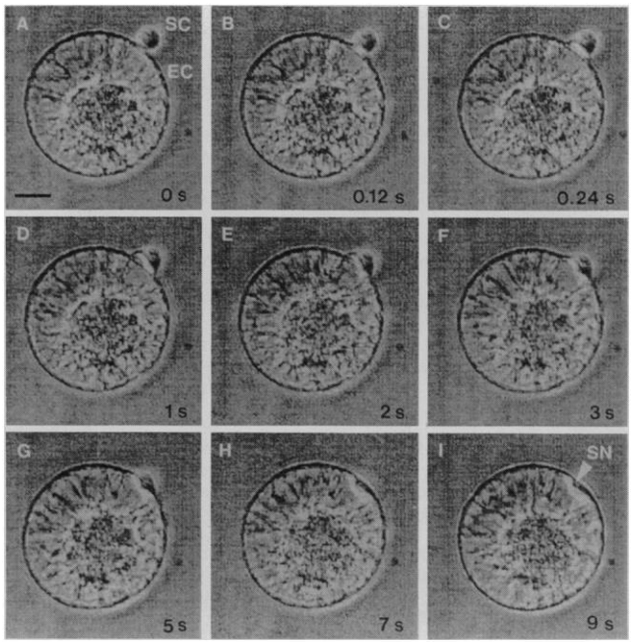

Fig. 1. Phase contrast micrographs of in vitro gametic adhesion and fusion in maize. Isolated sperm cells (SC) and egg cell protoplasts (EC) were placed in contact in a fusion medium containing 5 mM $CaCl_2$. After 1 min 50 s for adhesion (**A**), the gametes fused (**B** to **I**). The fusion sequence was recorded as described (*23*). Pictures were taken at varying time intervals over a period of up to 9 s, as indicated in lower right corners. After fusion (I), the sperm nucleus (SN) is still visible within the egg cell protoplast. Bar, 15 μm.

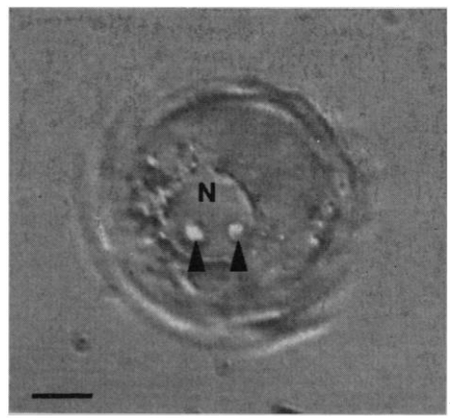

Fig. 3. Phase contrast micrograph of a fusion product kept at room temperature (22° to 24°C) for approximately 20 hours in 5 mM $CaCl_2$. Two nucleoli (unlabeled arrowheads) are visible within the nucleus (N). Bar, 10 μm.

the fusion event, the fusion products underwent a transient roughening and contraction. Fusion of central cell protoplasts with sperm cells in 5 mM $CaCl_2$ occurred in less than 1 s—more rapidly than did fusion of sperm-egg pairs (*25*).

Gametes fused in 5 mM $CaCl_2$ were stained with the fluorochrome DAPI (Fig. 2) (*26*). The male nucleus was incorporated in the female gamete and migrated toward the egg nucleus, as observed after electrofusion (*9*, *10*). About 20 min after gamete fusion, the male nucleus became difficult to see in the egg cell cytoplasm. Using phase-contrast microscopy, we observed the nucleus of a fusion product with two nucleoli after approximately 20 hours at 24°C in the fusion medium (Fig. 3). This is also observed in vivo after the fusion of the male and female nuclei (*27*) and thus strongly suggests that karyogamy occurs in the in vitro fusion system.

In order to determine whether fusion of a male gamete with a female gamete is specific, we performed the fusion trials with isolated sperm cells, egg cell protoplasts, and mesophyll protoplasts (*28*) in all combinations (Table 1). These experiments were performed in 5 mM $CaCl_2$, the concentration at which the highest percentage of sperm-egg fusions was obtained. Under these conditions, we observed adhesion in each situation. Fusion, however, only occurred in sperm-sperm pairs (16.7%) and once in a sperm-mesophyll protoplast trial after 1 hour adhesion (Table 1). Thus, fusion was mainly restricted to sperm-egg pairs. In order to determine whether polyspermy is possible, additional male gametes were moved into contact with fertilized eggs in 5 mM $CaCl_2$. Although sperm cells and fertilized eggs adhered, no fusion occurred in 20 trials over periods ranging from 10 to 45 min after the initial fusions. These results suggest that a block to polyspermy exists at the gamete level in flowering plants, as is the case in mammals (*5*).

The medium we used for gamete fusion has a calcium concentration between 1 and 10 mM, and the question arises whether these concentrations are close to those of the in vivo conditions. Although uncertain, it is considered that the concentration of extracellular free calcium ranges from 10 μM to 10 mM for plant cells (*29*). In addition, millimolar calcium concentrations are beneficial for plant protoplast culture (*30*), but the effects of calcium on the survival of isolated sperm cells are contradictory (*31*). Although millimolar concentrations of extracellular calcium are required for sperm-egg fusion in mammals (*2*, *24*), little is known about calcium requirements for gametic fusion in flowering plants. Studies on embryo sacs by x-ray analysis, antimonate precipitation assays,

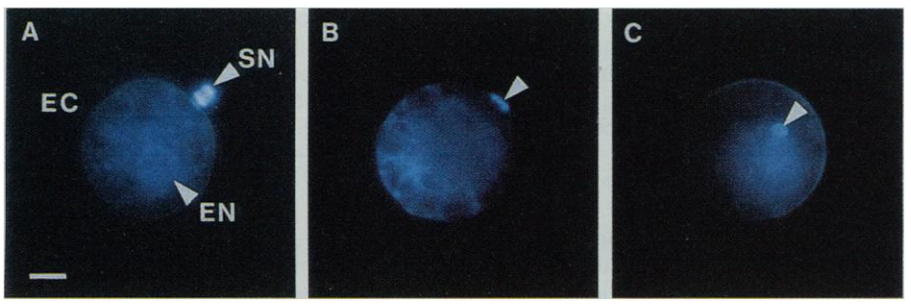

Fig. 2. Fluorescence micrographs of in vitro gametic adhesion and fusion in maize. The protoplasts were stained with the fluorochrome DAPI (*26*) during adhesion of gametes in 5 mM $CaCl_2$ (**A**), 3 min after fusion (**B**), and 12 min after fusion (**C**). The sperm nucleus (SN and unlabeled arrowheads) is integrated into the egg cell protoplast (EC). It migrates toward the cluster of egg cell organelles that includes the egg cell nucleus (EN). Bar, 15 μm.

Table 1. Specificity of fusion. Mesophyll protoplasts, egg cell protoplasts, and sperm cells were isolated and pairs put into contact in 5 mM $CaCl_2$. Percent indicates the percentage of fused pairs, and numbers in parentheses indicate the number of trials. Fusion was mainly restricted to egg-sperm pairs under our conditions. Note that 79.7% of initial sperm-egg pairs fused. Up to 96.9% of eggs fused after two new attempts with added sperm cells when no fusion was seen to occur with the first sperm cell.

	Isolated egg cell protoplast (%)	Isolated mesophyll protoplast (%)	Isolated sperm cell (%)
Isolated sperm cell	79.7 (64)	1.8 (57)	16.7 (24)
Isolated mesophyll protoplast	0 (12)	0 (20)	
Isolated egg cell protoplast	0 (17)		

and fluorescent probes like chlorotetracycline show that the local concentration of bound calcium within the synergids is high when compared with that in adjacent cells (*32, 33*)—in particular, in the synergids of grass species such as wheat and pearl millet (*33*). The sperm cells are released from the pollen tube into a degenerating synergid before fusion with the egg and central cells, so the calcium concentration in the medium surrounding the fusing gametes may be high, and our in vitro conditions may thus reflect those in vivo conditions.

Under our conditions, fusion seems to be mainly restricted to sperm and egg cell protoplasts. This fusion specificity suggests that cell recognition is preserved. Thus, this kind of in vitro fusion system should allow studies of gametic recognition, barriers to cross-species fertilization, the mechanism of gametic fusion, and intracellular signals after fusion. These experiments also provide the possibility of investigating the cellular and molecular biology of the single cell zygote. In particular, the integration of the male nucleus and cytoplasm, and other postfertilization events, such as modifications to the cytoskeleton, can be studied.

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14. Maize plants (*Zea mays* L.) of inbred line A188 were grown in a regulated growth chamber at the Université Claude Bernard, Lyon, France. The temperature ranged from 25°C (day) to 19°C (night), with a 16-hour photoperiod at 70 to 90% relative humidity and a light intensity of 700 to 750 μmol m^{-2} s^{-1}, provided by high-pressure sodium lamps (Sodiclaude 1000 W, Claude, Tinen, Belgium). To prevent pollination, the ears used for female protoplast isolation were bagged before silk emergence. The ears were collected at a receptive stage when the emerged silks were 13 to 14 cm long.
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17. Female gametes were isolated as follows: Ovule pieces containing embryo sacs were removed from the ovaries and incubated in 1% pectinase (Serva, Heidelberg, Germany), 0.33% pectolyase (Sigma Chimie, St. Quentin Fallavier, France), 0.67% macerozyme Onozuka R10 (Yakult Honsha, Tokyo, Japan), and 0.67% cellulase Onozuka R10 (Yakult Honsha), pH 5.0, adjusted to 550 mosM with mannitol. Ovule pieces were treated for about 25 min at room temperature. Egg cells and central cells were then isolated from the ovule pieces by microdissection with glass microneedles (*21*) under an inverted IM 35 Zeiss microscope (Oberkochen, Germany).
18. The pH was 6 to 6.5. We measured pH using Neutralit pH indicator strips (Merck, Darmstadt, Germany).
19. The fusion medium was very slightly diluted with 0.5 M mannitol when the gametes were introduced into it. Probably up to 1/20th of the fusion droplet volume was added with the gametes.
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22. We define adhesion as a step during which gametes appear to be attached and can be moved together.
23. We recorded the fusions with a Sony DXC-107P charge-coupled device color video camera (Sony) mounted on the inverted microscope and with a Panasonic NV-FS 100 HF videocassette recorder (Matsushita Electric Industrial, Osaka, Japan). We obtained pictures by digitizing the video recordings with a Macintosh IIci computer (Apple, Cupertino, CA) equipped with a 24 MxTv video digitizing board (Rasterops, Santa Clara, CA) and then printing the pictures with a Personal Laserwriter (Apple).
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26. DAPI (4′, 6-diamidino-2-phenylindole) is a fluorescent binding probe for DNA. We prepared a staining solution of 14 μM DAPI (Sigma Chimie, St. Quentin Fallavier, France) in 0.5 M mannitol. The apposed gametes and the fusion products were introduced in staining solution droplets of about 3 to 4 μl by use of the microcapillary transfer system (*21*) and observed under an inverted IM 35 microscope equipped for fluorescence study.
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Biodegradable Long-Circulating Polymeric Nanospheres

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Injectable nanoparticulate carriers have important potential applications such as site-specific drug delivery or medical imaging. Conventional carriers, however, cannot generally be used because they are eliminated by the reticulo-endothelial system within seconds or minutes after intravenous injection. To address these limitations, monodisperse biodegradable nanospheres were developed from amphiphilic copolymers composed of two biocompatible blocks. The nanospheres exhibited dramatically increased blood circulation times and reduced liver accumulation in mice. Furthermore, they entrapped up to 45 percent by weight of the drug in the dense core in a one-step procedure and could be freeze-dried and easily redispersed without additives in aqueous solutions.

The development of intravenously administered carriers with blood circulation times long enough to continuously deliver drugs, imaging agents, or other entities to specific sites of action has been a major challenge. The desired features of such a carrier include (i) that the agent to be encapsulated comprises a reasonably high weight fraction (loading) of the total carrier system (for example, more than 30%), (ii) that the amount of agent used in the first step of the encapsulation process is incorporated into the final carrier (entrapment efficiency) at a reasonably high level (for example, more than 80%), (iii) the ability to be freeze-dried and reconstituted in solution without