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H. Q. Tian · S. D. Russell

Micromanipulation of male and female gametes of *Nicotiana tabacum*: II. Preliminary attempts for in vitro fertilization and egg cell culture

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Abstract This research is part of an attempt to establish an in vitro fertilization system in tobacco to aid in understanding mechanisms of fertilization. Fusions of isolated male and female gametes were induced in a polyethylene glycol solution. Fusion appears similar to that in maize. One nuclear division of both an unfertilized egg cell and a synergid was induced in KM8p medium with 1 mg/l 2,4-dichlorophenoxyacetic acid in a microchamber culture; one cellular division of the egg cell was also induced in the same medium in solid-drop culture. The osmolality of suspension culture feeder cells was critical for the development of these cells. These results indicate that in vitro fertilization is possible in tobacco, which would be the first such system in dicots.

Key words In vitro fertilization · Egg cell · *Nicotiana tabacum* · Polyethylene glycol · Sperm cell

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *PEG* Polyethylene glycol

Introduction

Fertilization in flowering plants is an important process during plant development. In the past, research into the mechanisms of double fertilization has been limited to structural observations of in vivo events because the egg cell is an integral part of the female gametophyte and is located deep within the ovule and gynoecium. Recently, however, gametes have been isolated more routinely and have been fused using an in vitro system, leading to the formation of artificial zygotes in maize (Kranz et al. 1991a; Faure et al. 1994) and wheat (Kovács et al. 1995), and regeneration of fertile plants in maize (Kranz and Lörz 1993). This technical development not only establishes an in vitro system for examining fertilization events and embryo development of higher plants, but also provides a new method for wide hybridization through the fusion of naturally occurring haploid cells, which is of great potential value in genetic manipulation (Yang and Zhou 1992).

For in vitro fertilization, three basic techniques need to be perfected. (1) Isolation of male and female gametes. Both the quantity and quality of the gametes must be assured, especially for female gametes. (2) Microfusion of the gametes. To ensure that fusion occurs exclusively between egg and sperm cells, fusion is carried out using only a defined pair of cells, which requires special equipment or special micromanipulation skill. (3) Microculture of fusion products. Because the number of fusion products is limited, a special feeder system is necessary for further in vitro development. These three basic techniques form the entire manipulation system required for in vitro fertilization. Combining these basic techniques and recognizing the special nature of gametes render the situation significantly different from that of somatic hybridization. The significance of establishing an in vitro fertilization system exceeds the realm of fertilization research itself, since it also represents a new threshold in plant cell engineering.

As outlined previously, tobacco is a model plant for plant tissue culture and somatic engineering, and well-developed protocols are available for isolating male and female gametes (Tian and Russell 1997). In this paper, we present the elements of such an in vitro fertilization system in tobacco, which is the first dicot flowering plant to be examined in this manner.

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H. Q. Tian[1] · S. D. Russell (✉)
Department of Botany and Microbiology,
University of Oklahoma, Norman, OK 73019, USA
Fax no.: +1-405-325-7619
email: srussell@ou.edu

Present address:
[1] Department of Biology, Wuhan University, Wuhan, China

Materials and methods

Plants of *Nicotiana tabacum* L. were grown in a controlled condition as previously, and gametes were isolated as described in Tian and Russell (1997).

Fusion of male and female gametes was induced using polyethylene glycol (PEG, average molecular weight 3350). The fusion solution contained 5 mM $CaCl_2$, 5 mM KH_2PO_4 and 3 mM 2-(N-morpholine)ethanesulfonic acid; mannitol was varied between 0 and 6%, and PEG-3350 was varied between 5% and 25%. About 10 μl of fusion solution was placed on a slide and covered with mineral oil. One egg cell and several sperm cells were collected and transferred into the fusion solution using a microinjector (Type 5242, Eppendorf, Germany). The cells were brought into contact for 6–12 min and then transferred into a solution containing a lower concentration of PEG. Finally, the fusion product was transferred into a KM8p medium (Kao and Michayluk 1975) containing 3% sucrose and 6.5% glucose.

Embryo sac cells are always limited in number, and therefore a microculture technique was used to obtain cell division. Egg cells and synergids were cultured using both a microchamber and solid-drop culture technique with suspension culture cells of tobacco used as feeder cells. For microchamber culture, we used a 3.5-cm plastic dish containing solid agar with a 0.5-cm cavity in the center. The egg cells were cultured in the cavity with 0.05 ml medium (450–550 mosmol/kg H_2O) and 1.5 ml of suspension culture cells (310–330 mosmol/kg H_2O) carefully poured around the outside. For solid-drop culture, embryo sac cells were embedded in 0.02 ml of medium containing 1.5% agarose (ultralow gelling temperature, type IX, Sigma), solidified for 10 min at 5°C, and surrounded by the feeder suspension.

For embryo sac cell culture, KM8p medium was supplemented with 0.5 to 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose and 6–8% glucose. Suspension culture cells were maintained in B_5 medium (Gamborg et al. 1968), supplemented with 1 mg/l 2,4-D, 3% sucrose, and 3% glucose. The suspension culture cells were obtained by putting tobacco anther callus grown on MS agar into liquid B5 medium (shaking at 30 rpm at 25°C in the dark); these cells were subcultured weekly for up to 5 months.

Results and discussion

Fusion of male and female gametes

In inducing fusion of male and female gametes, the osmolality of the fusion solution and the concentration of PEG are critical. In conditions of low osmolality (15–20% PEG, 180–280 mosmol/kg H_2O), sperm cells became swollen and broke more easily than egg cells. In conditions of high osmolality (30% PEG, 620 mosmol/kg H_2O), however, egg cells shrank more readily than sperm cells. The viscosity of 30% PEG solution also makes fusion difficult, because of difficulties in moving the gametes into contact. A suitable osmolality for the fusion solution must be selected to meet the requirements of both the egg and sperm cells. In the 5% PEG fusion solution (supplemented with 7% mannitol, 500 mosmol/kg H_2O), no fusions occurred even when sperm and egg cells were brought into contact for more than 20 min. When the PEG was increased to 15% (supplemented with 4.5% mannitol, 510 mosmol/kg H_2O), a sperm cell and an egg cell fused after 10 min of cell contact (Fig. 1a). The fusion procedure itself was very quick and generally took less than 3 s. The entire cytoplasm of the sperm cell with its nucleus fused with the egg to form a combined cell. At high magnification, the fusion products are often seen to be binucleated and contain a large nucleus, presumably from the egg cell, and a small one, from the sperm (Fig. 1b).

Interestingly, in the above-mentioned fusion solution, the fusion of egg cells with other egg cells and sperm cells with other sperm cells was more rapid; only 3 min was usually needed from cell contact to fusion (Fig. 1c).

Fusion products that were transferred directly from the fusion solution to the culture medium containing 9% glucose did not survive well in culture. Therefore, fusion products were usually transferred first into a solution with a low concentration of PEG (5% PEG and 7% mannitol) for about 10 min, and then introduced into a KM_8p medium. Using this technique, the fusion products remained in good condition for at least 1 day. Egg cells already in adhering to a sperm cell in PEG could be transferred into a lower PEG solution without inhibiting cell fusion and then transferred into the culture medium. Sufficient time must be allowed for adherence though, because egg-sperm cell pairs that are transferred too early can separate.

We also tried to fuse egg cells and sperm cells in a solution of high calcium (10–50 mM) and high pH (9–10) similar to the protocol of Kranz and Lörz (1994), but no fusion occurred, even after 30 min of contact between the sperm and egg cell.

Fusions were also obtained using PEG between a pair of synergid cells (Fig. 1d), one central cell and an egg cell (Fig. 1e), and a pair of central cells (Fig. 1f). The larger size of these cells facilitated fusion. Attempts to culture the fusion products are underway, but are limited by the need to avoid contamination at each of the many transfer steps.

The fusion of male and female gametes in vitro represents an opportunity to provide experimental insights into the mechanisms of fertilization. To date, a number of methods have been used to fuse the male and female gametes, but only two plants, both monocots, have been used successfully: maize and wheat. Several methods have been used to obtain this fusion. In vitro fusions were first performed in maize using electrofusion (Kranz et al. 1991a), and these could be achieved using nearly any conceivable combination of gametophytic and sporophytic cells (Kranz et al. 1991b). Wheat gametes have also been combined using this method (Kovács et al. 1995). High calcium concentrations (50 mM $CaCl_2$) and high pH (pH 11.0) have been successful in inducing fusion in maize (Kranz and Lörz 1994). Faure et al. (1994) used an approach of combining adherent sperm and egg cells in a minimal solution containing 1, 5, and 10 mM $CaCl_2$ and found a requirement for calcium during gamete fusion with an optimum of 5 mM $CaCl_2$. Fusion was highly successful in sperm-egg cell pairs (79.8%), whereas only 1.8% of sperm-mesophyll protoplast combinations fused under these conditions. This specificity of fusion has been interpreted as a potential sign of gametic cell recognition (Faure et al. 1994). Fusion among gametophytically derived cells of tobacco also occurred more quickly than fusion involving somatic (mesophyll) protoplasts, suggesting possible differences in the

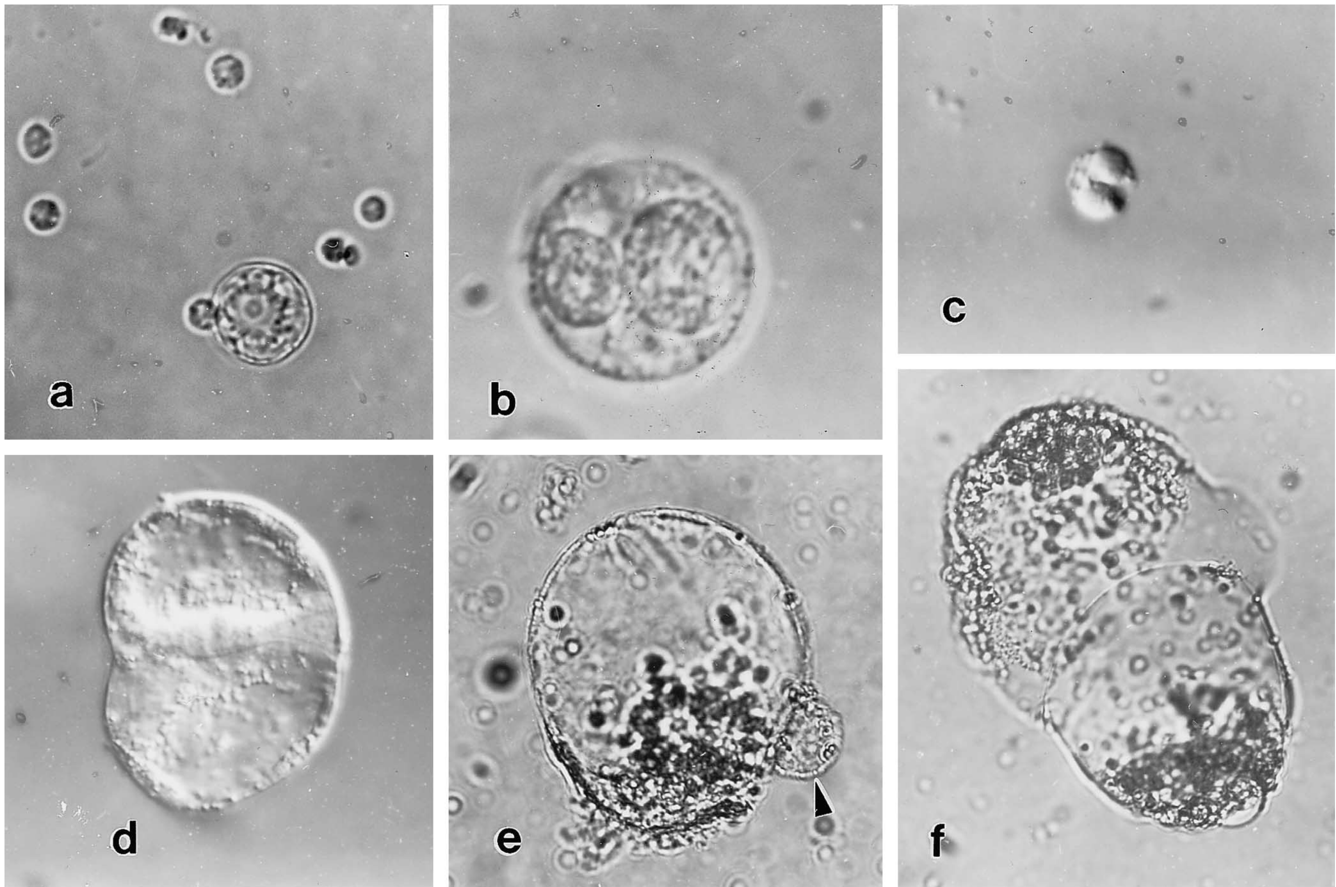

Fig. 1a–f Egg and sperm cell fusion. **a** Egg cell and adherent sperm cell. Other sperm cells are also present in medium. ×500. **b** Fusion product of egg and sperm cell. The egg nucleus (right) is larger. ×1600. **c** Fusion product of two sperm cells. ×950. **d** Fusion of two synergid cells. ×950. **e** Fusion of egg cell (*arrowhead*) and central cell. ×300. **f** Fusion of two central cells. ×300

condition of the plasma membranes of gametoplasts and somatic protoplasts (Sun et al. 1995). We observed a similar tendency of reproductive cells to fuse more readily. The time between adhesion and fusion of tobacco egg and synergid cells was only 3 min. In the fusion of egg and sperm cells, however, more than 10 min was required for adhesion to occur prior to fusion. This difference in the rapidity of fusion in PEG also suggests that significant plasma-membrane-based differences may occur between cells, and that these may be related to the natural propensity of sperm cells to fuse with egg cells.

Egg cell and synergid culture

In culturing egg cells and synergids, the osmolality of the medium appears to be critical, because the feeder cells, egg cells, and synergids had different optimum osmolalities. When the osmolality of the medium was less than 450 mosmol/kg H_2O, the cells of the embryo sac swelled and through budding formed several small subprotoplasts. This phenomenon was even more evident in synergids than in egg cells. If the osmolality of the medium rises above 550 mosmol/kg H_2O, the egg cells appear to shrink more than synergids. This reflects different osmotic requirements of the egg and synergid cells that must be addressed in culture. In microchamber cultures, after 2 days of incubation, the nuclei of egg cells (20%) divided in a medium of 460–480 mosmol/kg H_2O (Fig. 2a,b), whereas the nuclei of synergids (37%) divided in a medium of 530 mosmol/kg H_2O (Fig. 2c, d). None of the cells divided outside of its specific osmotic optimum.

In solid-drop culture, some egg and synergid cells changed shape from round to ellipsoidal after 1 day in culture and displayed numerous starch grains surrounding the nucleus and in the periphery of the cytoplasm (Fig. 2e, f). After 3 days in culture, some ellipsoid cells divided to form a small apical cell and a larger basal cell (Fig. 2g); however, if the cells do not become ellipsoid, the nucleus divides once, and cytokinesis does not occur.

In our experiments, we found that suspension culture feeder cells often displayed plasmolysis and stopped growing when the osmolality of the medium neared the optimum for embryo sac cells (above 450 mosmol/kg H_2O). After 5 months of suspension culture in which we selected for cells tolerant of higher osmotic conditions, the optimum osmolality of the suspension culture was increased from 150 mosmol/kg H_2O to an optimum of 320–340 mos-

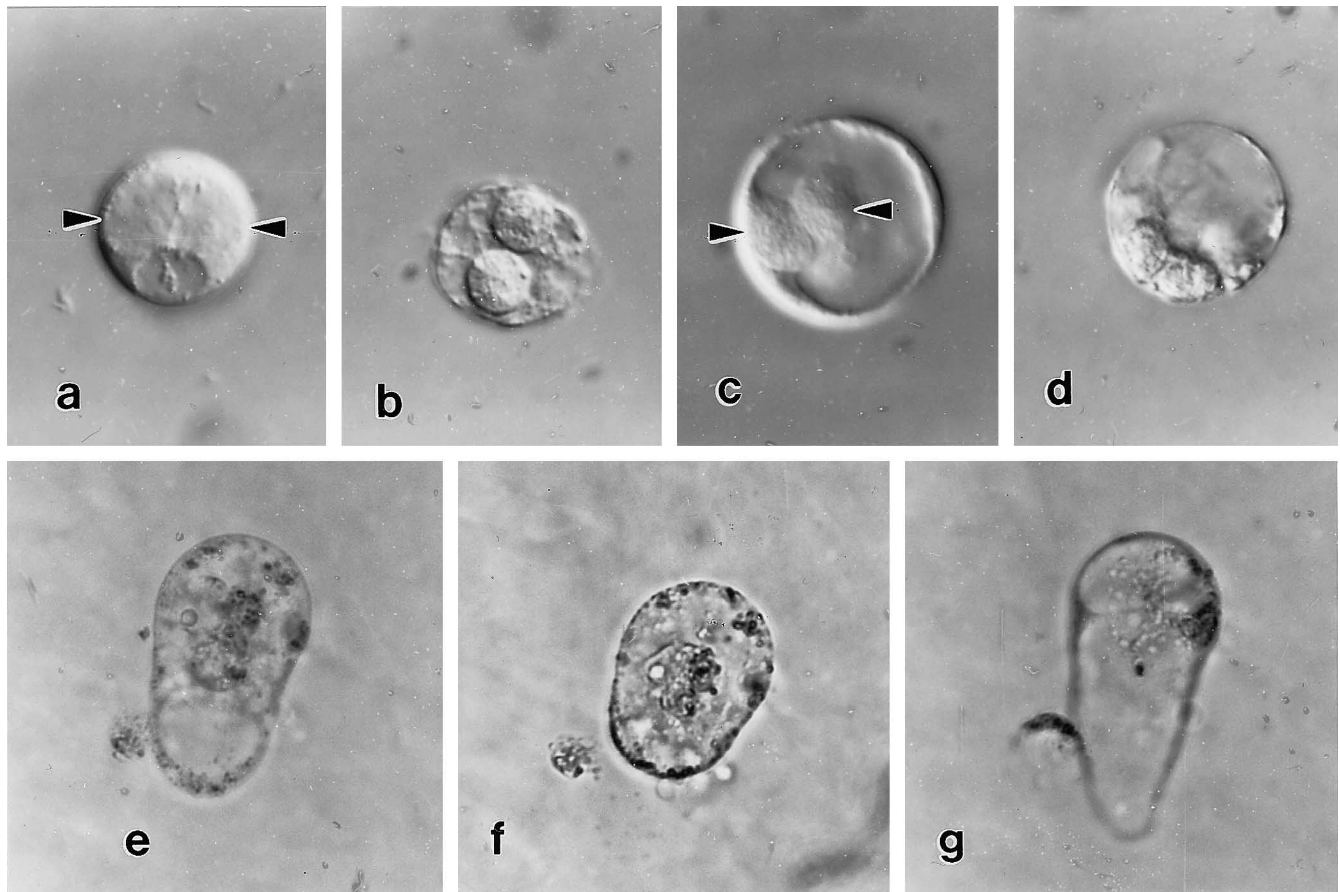

Fig. 2a–g Egg cell and synergid culture. **a, b** Nuclear division (*arrowheads*) of egg cell after 2 days in microchamber culture. ×950. **c, d** Nuclear division (*arrowheads*) of synergid cell after 2 days in microchamber culture. ×950. **e** Synergid cell becomes ellipsoid after 1 day in solid-drop culture. ×500. **f** Nuclear division of cell shown in **e** occurs after 2 days in culture. ×500. **g** After 3 days in culture, an egg cell divides and forms a small apical cell and larger basal cell. ×500

mol/kg H_2O. Since their optimum osmolality still differed from embryo sac cells, the feeder cultures of both microchamber and solid-drop cultures had to be maintained in a separate compartment with different osmotic conditions. To reduce evaporation of water from the microchamber and solid-drop cultures and to reduce the opportunity for contamination, the feeder suspension cells were placed in the plastic dish at the same time as egg apparatus cells; these normally did not leak into the microchamber and solid-drop until 2 days later. In this manner, egg apparatus cells retained their viability for 5 days, as indicated by organelle movement. Suspension cultures will continue to be selected until cells tolerant of the osmotic conditions required by gametes are obtained.

Hormone treatments of 0.5 mg/l 2,4-D were insufficient to induce nuclear division in either the egg cell or synergid, and when this was increased to 2 mg/l 2,4-D, the cultured cells broke down easily and were quickly lost. Successful nuclear division and cytokinesis in microchamber and solid-drop cultures were obtained only in media containing 1 mg/l of 2,4-D.

Fertile plants have been regenerated from in vivo-produced isolated zygotes using barley (Holm et al. 1994), maize (Leduc et al. 1996), and wheat (Holm et al. 1994), as well as in-vitro-produced zygotes of maize (Kranz and Lörz 1993). A microcallus was produced from in-vitro-derived wheat zygotes (Kovács et al. 1995). Using tobacco, only a single division was obtained when in-vivo-fertilized zygotes were cultured (Fu et al. 1996). Unfertilized egg cells of barley and wheat in vitro did not divide (Holm et al. 1994). Maize egg cell cultures (line Garant) yielded 6% egg cell division when a 1-h treatment of 25–40 mg/l 2,4-D was used before culture, but without the 2,4-D treatment, the egg cell did not divide (Kranz et al. 1995). In our experiments with tobacco, egg cells incubated using 1 mg/l 2,4-D in microchamber culture underwent only mitosis, whereas similar egg cells cultured using the solid-drop method underwent both mitosis and cytokinesis. The formation of ellipsoid egg cells only occurred under the latter conditions and may be critical for continued development.

Interestingly, when egg cells and synergid cells were cultured in the same medium in microchamber culture, there were different requirements for osmolality between the egg cell and synergid cells. In a low-osmolality medium (460–480 mosmol/kg H_2O), the nuclei of egg cells sometimes divided, whereas synergids budded into sev-

eral small subprotoplasts. In a high-osmolality medium (520–540 mosmol/kg H_2O), the nuclei of synergid cells sometimes divided whereas the egg cells shrank. This may reflect a difference in physiological state between the egg cell and synergid cell that may be potentially useful in selecting gametophytic cell types in culture.

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