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Isolation of living sperm cells and *in vitro* fusion of *Torenia fournieri* gametes

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Summary: In contrast to most angiosperms, Torenia contains a naked embryo sac and therefore has been considered since many years as an exciting model plant to study the double fertilization process of flowering seed plants. It is thus not surprising that the isolation of protoplasts from the female gametophyte has been reported already 20 years ago by Mol, the isolation of megaspores and megagametophytes has been published by the authors of this manuscript in 1996 and in 1999. The isolation of the male gametophyte and of sperm cells was published by the authors in 2004. The isolation of viable *Torenia* sperm cells is a crucial part of the elaboration of an *in vitro* fertilization system. *Torenia* sperm cells were isolated from in vivo – *in vitro* cultured pollen tubes. In this system pollen tubes first grow inside a cut style then follow their elongation in a solid isolation medium. The medium contained agarose in order to detain pollen tube contents. Released sperm cells and enzymatically isolated egg cells were collected and handled using glass micropipettes and transmitted to an electrofusion apparatus or polyethylene glycol containing media for fusion probes.

Key words: Sperm cell; Egg cell; Electrofusion; Micromanipulation; *In vitro* fertilization; *Torenia*.

Introduction

There have been many attempts to establish complete *in vitro* fertilization systems in different plant species. Most of the first successful *in vitro* fertilization experiments have been done on monocots (*Kranz* et al., 1991a, 1991b; *Theunis* et al., 1991; *Kranz & Lörz,* 1993; *Faure* et al., 1994; *Kovacs* et al., 1994; *Kranz* et al., 1995; *Kranz & Dresselhaus,* 1996; *Yang & Zhou,* 1998; *Faure,* 1999).

There is little information regarding the isolation of male gametes of dicotyledonous species with bicellular pollen (*Mo & Yang* 1992; *Sun* et al 2000). The most relevant difference between bicellular and tricellular pollen is the site and manner of sperm cell formation. Sperm cells of plant species with tricellular pollen can be isolated by making the pollen grains burst by osmotic shock or grinding (*Cass* 1973; *Russell & Cass* 1981; *Matthys-Rochon* et al. 1987). In *Torenia*, *Nicotiana* and other dicot species sperm cell formation takes place in growing pollen tubes within the style which makes sperm cell isolation arduous. Semi *in vitro* systems are in use to obtain sperm cell containing, uncovered pollen tubes (*Tian & Russell* 1997a, 1997b; *Higashiyama* et al., 1997; *Higashiyama* et al 1998, 2001; *Higashiyama,* 2002; *Vági* et al., 2004). Sperm cell isolation is commonly carried out via osmotic shock in liquid media. Pollen tubes burst at their tips, and the sperm cells embedded in the vegetative cytoplasm released into the isolating solution and become dispersed immediately. The cytoplasm of the vegetative cell is firmly attached to the male germ unit and form a viscous aggregation that is complicated to use for *in vitro* fusion experiments.

Torenia fournieri was the source material of our experiments. We have previously reported several experiments on the development and isolation of the female and male gametophyte of *Torenia* (*Kristóf & Imre,* 1996; *Imre & Kristóf,* 1999; *Vági* et al., 2004). Our recent studies served as a good basis for establishing a complete *in vitro* fertilization system for *Torenia*. This work deals with the isolation of viable sperm cells of *Torenia*, the electrofusion and PEG induced fusion of isolated egg cells with single sperm cells.

Materials and methods

Plants of *Torenia fournieri* were grown in a controlled growth chamber at a temperature of 20 °C (night) and 24°C (day) with 14 h photoperiod.

Isolation of egg cells

The egg cell isolation procedure was based on the method developed by *Imre and Kristóf* (1999). The following modifications were needed to obtain high quantity of viable egg cells. The isolation solution consisted of 10 % (v/v) modified Nitsch 10x stock solution (Higashiyama et al. 1998), 2 % sucrose and different concentrations of mannitol ranging from 4 % to 10 %, 2 % cellulase Onozuka R-10 (Yakult Honsha), 1 % macerozyme Onozuka R-10 (Yakult Honsha). The pH of the isolation solution was adjusted to 5.0.

Sperm cell formation and isolation

Torenia pollen is shed in a bicellular condition. Sperm cell formation takes place in growing pollen tubes in the style 4 hours after pollination (*Vági* et al., 2004). We improved a

semi *in vitro* method that was based on the method of *Higashiyama* et al. to obtain viable sperm cells (*Higashiyama* et al., 1998). Styles were cross pollinated soon after dehiscence. We germinated pollen and allowed it to grow on the stigma using the semi *in vitro* method to obtain pollen tubes in which sperm cells had formed. After 4 hours of growth in vivo styles were excised 16-18 mm from the tip of the stigma without damaging the pollen tubes. The cut end of the style was then immersed in a modified Nitsch culture medium (*Higashiyama* et al. 1998) containing different concentrations of low gelling temperature agarose Type VII (Sigma A4018) ranging from 0.1 % to 3 %. Pollen tubes of *Torenia* arrive at the micropyle 8–10 hours after pollination in vivo. The phase in the cell cycle of gametes, and consequently the timing of sperm cell isolation might be a crucial point of *in vitro* fertilization. Sperm cell isolation was carried out prior to arrival of pollen tubes in the embryo sac. Pollen tubes emerging from the cut end of the style were burst via osmotic shock to release tube contents. The isolation medium was diluted by adding half-concentrated liquid medium. Vegetative and sperm cell nuclei were labeled with DAPI. The progress of isolation was observed using an Olympus IMT-2 inverted microscope and isolated male germ units and sperm cells were collected in glass microcapillaries made on Flaming/Brown micropipette puller Model P-97 (Sutter Instruments Co., USA) using Narishige micromanipulators mounted on the microscope. Viability of the isolated gametes was determined by fluorescein diacetate (FDA) accumulation (0.1 % FDA in acetone diluted 1:200 in the medium).

Microscopy

For microscopy, agarose drop samples containing pollen tubes, isolated male germ units and sperm cells were fixed in a solution of 4% glutaraldehyde buffered to pH 7.5 with 0.01 M Na-HEPES, and transferred to 1% OsO4 solution for postfixation. Samples were dehydrated in a graded ethanol series, then embedded in Unicryl resin (SPI Supplies, West Chester, USA) for light and electron microscopy. Light microscopy specimens were examined with an Olympus BH-2 epifluorescence microscope.

Electrofusion of isolated egg- and sperm cells

The fusion of single isolated male and female gametes of *Torenia* was carried out following the method of *Kranz* et al. (1991a, 1991b) with minor modifications. Fusion medium I contained 9 % mannitol (540 mosmol/l H_2O) in distilled water. Fusion medium II contained 8 % mannitol, 3 % BSA Fraction V (Serva) (520 mosmol/l H_2O). The pH of the fusion media was adjusted to 6.0. The electrofusion was carried out in 50 µl droplets placed on coverslips. As a result of applying the AC-field (1000 kHz, 40–50 V/cm) the selected single egg cell adhered to one of the electrodes and was moved to the selected sperm cell. After the AC-field induced alignment of the gamete pairs fusion was induced by a single DC-pulse (50 µs, 40 – 70 V/cm). The distance between the electrodes was approximately 100 µm. Fusion experiments were carried out with electrofusion apparatus (Krüss Biojet CF 50, Hamburg). The electrodes were made of surgical stainless steel needles.

Polyethylene glycol (PEG) mediated fusion of gametes

The enzymatically isolated egg cells were washed in 9 % mannitol solution for 8 – 10 minutes before being transferred into the fusion medium. Isolated male gametes were transferred directly to the fusion droplet to avoid damage and loss of sperm cells (*Figure 1 d*). The PEG induced fusion of egg and sperm cell pairs was performed in a solution containing 10 % (v/v) of modified Nitsch 10x stock solution (*Higashiyama* et al., 1998), 5 % sucrose, 15 % PEG 4000. 10 µl droplets were placed on cover slips and overlayered with paraffine oil. The selected gametes (*Figure 1 e*) were manually paired using a microcapillary (*Figure 1 f*). The fusion droplet was diluted by gradually injected modified Nitsch medium. After 10 minutes conditioning fusion products were transferred to a modified Nitsch medium containing FDA to determine their viability.

Results

In enzymatic egg cell isolation osmolarity of the isolation medium was the most critical point. The appropriate mannitol concentration was 4 % for older *Torenia* plants grown in our breeding chamber. The above mentioned modified egg cell isolation protocol was very effective, accordingly there was abundant supply of quality female gametes. The optimum agarose concentration was 0.3 %. At agarose concentrations higher than 0.3 % the medium became viscous so that maneuvering with the microcapillaries was obstructed. Pollen tubes emerged from the cut end of the style approximately 6 hours after pollination. After further 3.5–4 hours in culture the tubes reached 1.5–2 mm in length and the medium was diluted to burst the tubes to release sperm cells. At our first attempts the culture medium was diluted en masse by adding half strength liquid medium which caused pollen tubes burst simultaneously. This synchronous release of the tube contents made sperm cell collecting abrupt and those male germ units remained stationary for long time, appeared to coagulate. Further on we diluted the solid culture medium locally by pressing small quanta (1–2 µl) of half concentrated liquid culture medium into the solid medium near by the selected pollen tube tips. The injected liquid medium slowly diffused and only small cavities remained in which the pollen tube contents released. In this manner the solid medium anchored the male germ unit embedded in the vegetative cytoplasm (*Figure 1 a*) which facilitated sperm cell collection. Cells of the male germ unit were then separated by passing the isolated mass through the micropipette once or twice (*Figure 1 b*). Rarely sperm cells separated spontaneously or at least the cohesion within the

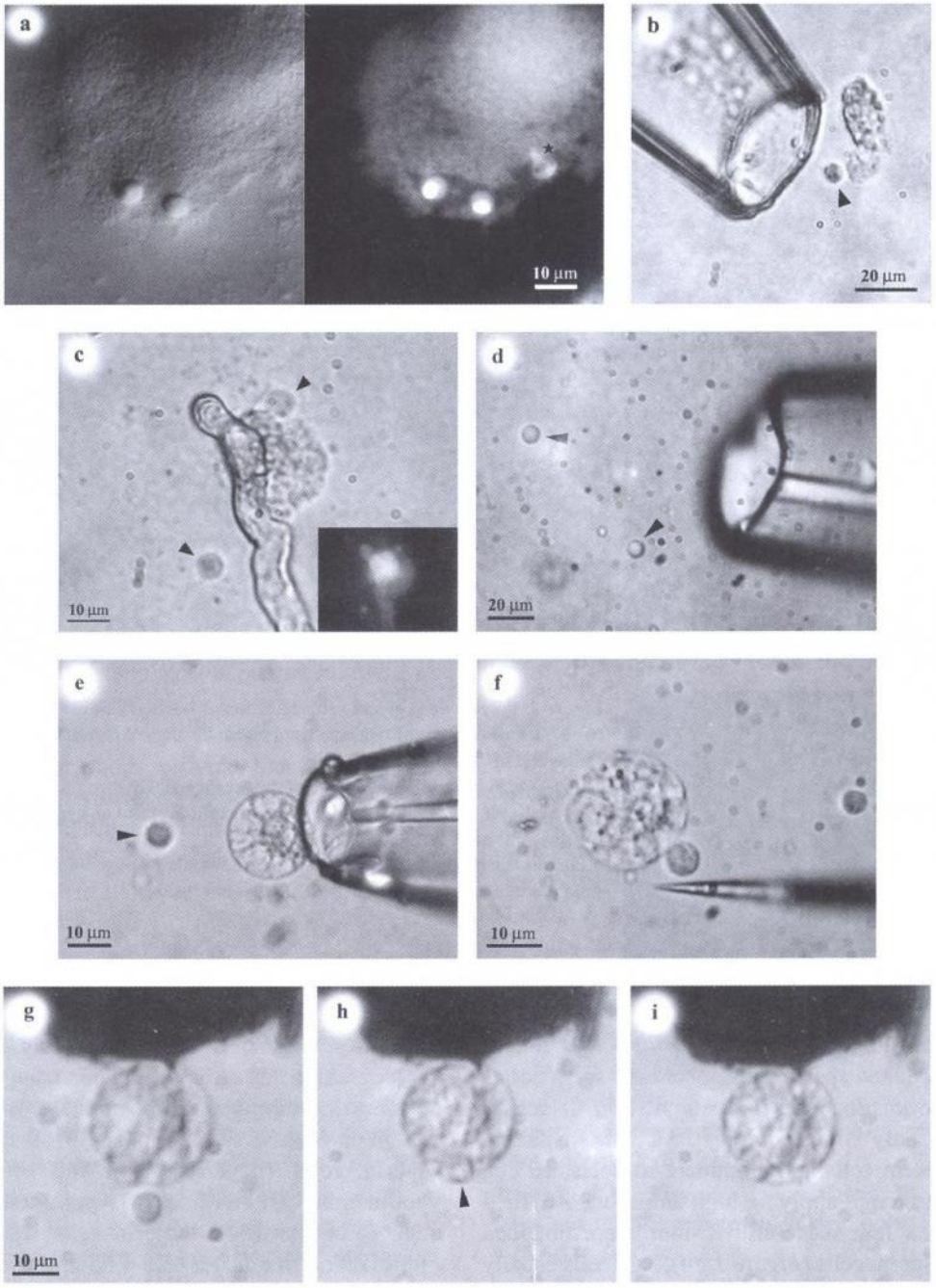

Figure 1. Sperm cell isolation and micromanipulation of isolated gametes. **a** Isolated male germ unit with its associated vegetative nucleus (*asterisk*) and the associated pollen tube cytoplasm. DIC microscopy *left*, DAPI epifluorescence *right*. **b** Separation of a single sperm cell from the male germ unit by passing the isolated mass through the micropipette. DIC. **c** Sperm cells (*arrowhead*) released spontaneously from the pollen tube. DIC and DAPI staining (*insert*). **d** Isolated sperm cells (*arrowhead*) transferred into the fusion droplet using microinjector. DIC. **e** A single isolated egg cell transferred to the fusion droplet containing a selected sperm cell (*arrowhead*). DIC. **f** Manual alignment of an egg and sperm cell using a microcapillary. DIC. **g – i** Electrofusion sequence of a pair of egg and sperm cell. *Arrowhead* indicates the fusion site. DIC.

male germ unit weakened while squashing through the flawed pollen tube wall (*Figure 1 c*). Sperm cells can be separated by washing soon after pollen tube burst. We found that continuity of the released male germ units come to stay in the course of time and those male germ units remained intact were impossible to dissolve through washing steps. Some of the tubes burst at areas other than the tip, and in these cases sperm cells were not recovered. Interestingly, isolated male gametes were not uniform in size and shape. Their diameter varied between 6–8 µm. Most sperm cells were spherical and there were a few oval or curved ellipsoidal ones.

In electrofusion induced fertilization of egg cells with sperm cells, the osmolarity and composition of the fusion solution both were critical. In fusion medium I containing only mannitol, isolated egg cells became fragile and often burst at higher voltage DC pulses even if osmotic properties were adequate. In fusion medium II containing BSA, egg

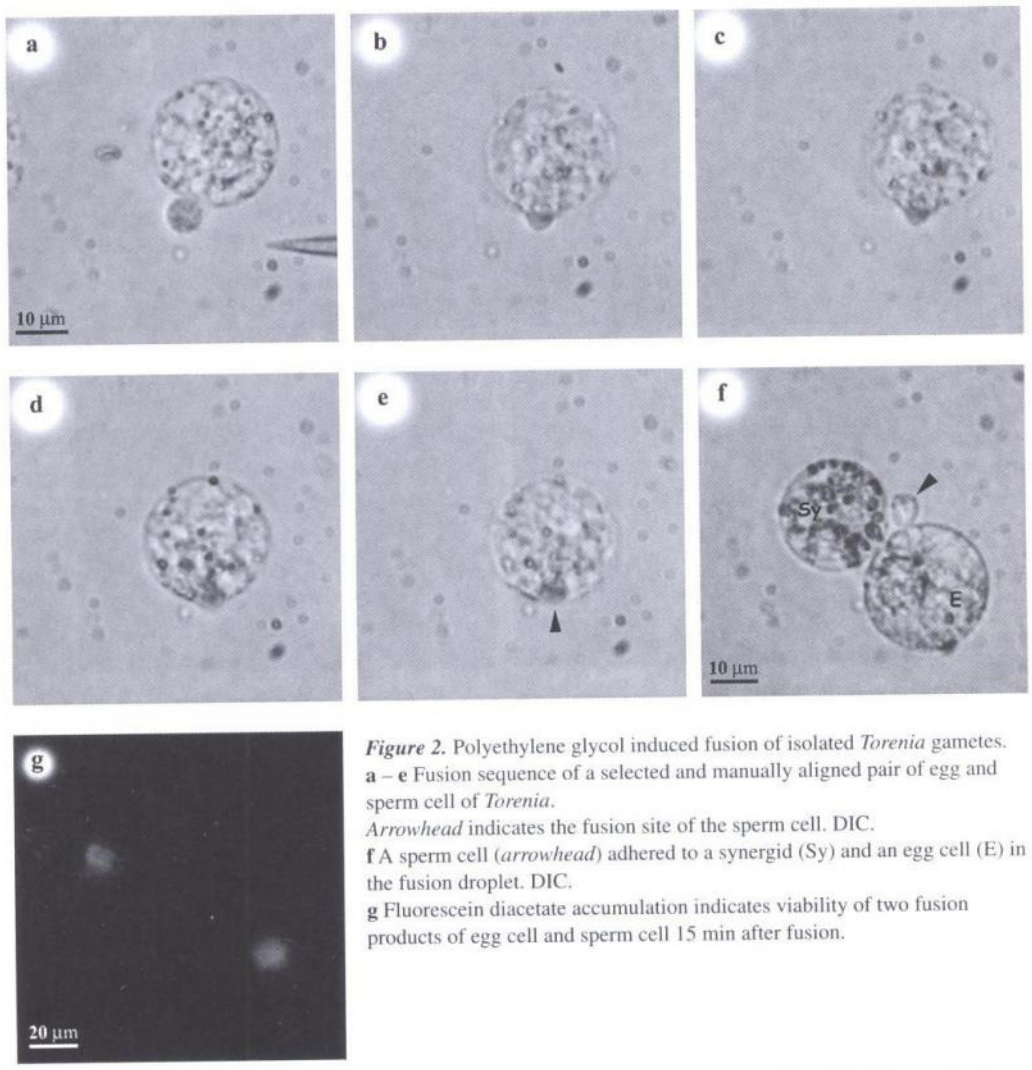

Figure 2. Polyethylene glycol induced fusion of isolated *Torenia* gametes.
a – e Fusion sequence of a selected and manually aligned pair of egg and sperm cell of *Torenia*.
Arrowhead indicates the fusion site of the sperm cell. DIC.
f A sperm cell (*arrowhead*) adhered to a synergid (Sy) and an egg cell (E) in the fusion droplet. DIC.
g Fluorescein diacetate accumulation indicates viability of two fusion products of egg cell and sperm cell 15 min after fusion.

cells were resistant to DC pulses up to 70 V for longer periods of time. Isolated sperm cells apparently were not as sensitive to osmotic properties or composition of fusion solutions supposedly due to the lack of vacuoles. Polarization of sperm cells and alignment to polarized egg cells was difficult though applying high amplitude AC-field (50 V/cm). Only a few successful fusions were obtained between egg and sperm cells (*Figure 1 g-i*). Egg cells usually burst with the DC-pulse before the completion of cell fusion. Egg cells fused more readily with synergids or even with other egg cells than with male gametes. Fusions between synergid cells were remarkably easy to perform.

Discussion

The above mentioned methods are evidently time consuming and requires micromanipulation skill, however provides exceptional control and makes sperm cells of one certain male germ unit distinguishable, which is an essential for ultrastructural examinations or *in vitro* fertilization probes to ascertain the presence of any preference in gamete fusion.

It is possible to obtain large number of egg cells and female gametophytes at various developmental stages in *Torenia fournieri*, as shown previously. *In vitro* fertilization at high efficiency or *in vitro* fusion using immature egg cells or megaspores might also be possible.

In one course of experiment 30–40 egg cells and 50–100 sperm cells (depending on the effectiveness of the pollination) can be obtained. Approximately 100 of mature egg cells and 150 of sperm cells was tried for *in vitro* fertilization in total. It take 4 hours for egg cells and 15–20 min for sperm cells from the start of isolation of gametes to *in vitro* fusion.

Fusions were obtained using polyethylene glycol between one egg and one sperm cell (*Figure 2 a-e*), pairs of synergid cells and pairs of egg cells. PEG-induced fusion eliminates the above described difficulties in polarization and electrofusion, risen from the significant size difference between female and male gametes, however there were differences in fusion speed of different cell combinations *(Fig. 2 f)*. Fusion products appeared to retain their viability for at least 15 min after the PEG induced fusion as indicated by fluorescein accumulation using the FDA viability assay (*Figure 2 g*).

Passiveness of male gametes in AC-field might be a consequence of the small size of *Torenia* sperm cells. The propensity of the synergids to fuse with any other protoplasts might be related to the diverse roles of the synergids in

reception of pollen tubes, fusion with the vegetative cell and the transmission of the two sperm cells to their targets, the egg and central cell. As a general rule, the larger cell size facilitated fusion as *Tian and Russell,* (1997a) reported in their work done on tobacco.

The fusion products other than fertilized egg cells e.g. two fused synergids are at best of theoretical importance since synergids presumably unable to divide due to their fate that is sealed in vivo. We plan to culture the fusion products of one egg and sperm cell and two egg cells in the near future.

We have established somatic and anther derived haploid suspension cultures for *Torenia*. Since the optimum osmolarity of suspension cultures significantly differs from egg cell derived fusion products it might be a time consuming work to select an adequately sucrose tolerant suspension culture.

In the future we have to combine *in vitro* methods with cellular and molecular analysis to monitor egg contraction, cell wall regeneration, karyogamy (emergence of two nucleoli in the zygote nucleus), the first presumably unequal division that may be observed specifically in the fertilized egg cell.

The above results are the completion of our recent studies to isolate male and female gametophytic cells of *Torenia*, which is the technical prerequisite for ultrastructural characterization of dicot gametes. These methods provide the keystone for establishing an *in vitro* fertilization system in a new dicot species which might facilitate understanding the hidden mechanisms and cytological aspects of double fertilization.

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