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Isolation of egg cells and zygotes of *Torenia fournieri* L. and determination of their surface charge

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Summary

Egg cells of *Torenia fournieri* were isolated from embryo sacs 1 day after anthesis using enzymatic digestion or mechanical dissection. About 5% of the egg cells and zygotes (2–3 from 50 ovules) could be mechanically dissected within 2 h. When 0.1% cellulase and 0.1% pectinase were added to the mannitol isolation solution, about 18% of the egg cells (8–10 from 50 ovules) could be isolated within 2 h. The egg cells isolated by mechanical dissection could be used for *in vitro* fertilization studies without any of the potentially deleterious effects of the enzymes on the plasma membrane of egg cell. The egg cells isolated using enzymatic digestion could be used in the study of the molecular biology of female gamete because more egg cells could be isolated with this technique. Using enzymatic digestion, over 10 zygotes from 50 ovules (over 20%) were isolated from the pollinated ovules. Coupled with our successful isolation of mature sperm cells, the isolation of egg cells of *T. fournieri* will make *in vitro* fertilization possible in a dicotyledon plant.

Keywords: Egg cell, Isolation, *Torenia fournieri*, Zygote

Introduction

Sexual reproduction is the primary means by which genetic diversity is introduced into populations of higher plants or animals. The union of genes that occurs during fertilization is a critical evolutionary mechanism and the means by which humans have improved cultivars through selective plant breeding. The location of the gametes, however, particularly the egg cell, within paternal or maternal tissue, restricts our ability to explore the fertilization process of higher plants. As a result, few detailed studies have been conducted that enhance our understanding of the fertilization process. The technique of *in vitro* fertilization, fusing isolated sperm and egg cells under controlled conditions, removes possible interference by somatic tissue, thereby providing opportunities for more detailed studies of angiosperm fertilization. Kranz *et al.* (1991) first fused the sperm and egg cells of maize to produce an 'artificial' zygote by electrofusion. Ultimately, this artificial maize zygote developed into a fertile plant (Kranz & Lörz, 1993). Now, *in vitro* fertilization techniques have been developed that can be used to study important applied reproductive topics or fields such as the molecular biology of male and female gametes, distant hybridization and cellular genetic engineering (Wang *et al.*, 2006). Until recently, the technique of *in vitro* fertilization has been successful only in maize and wheat, with most of the available data about zygote activation coming only from maize. Recent methodological advances have allowed for the application of *in vitro* fertilization to other, particularly dicotyledon, angiosperms (Wang *et al.*, 2006).

The isolation of egg cells is difficult in angiosperms because the egg cell is deeply embedded within the ovule. To date, egg cells of tobacco (Hu *et al.*, 1985), maize (Kranz *et al.*, 1991), petunia (Van Went & Kwee, 1990), perennial ryegrass (Van der Mass *et al.*, 1993), wheat (Kovács *et al.*, 1994), barley (Holm *et al.*, 1994) and rice (Zhang *et al.*, 1999) have been isolated. *Torenia fournieri* is a suitable species for fertilization research

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because its embryo sac protrudes partially through the micropyle and its egg cell, two synergids and part of the central cell can be clearly observed using light microscopy. Based on the unique embryo sac of *T. fournieri*, some studies on its *in vivo* fertilization mechanism had been reported (Higashiyama *et al.*, 1998, 2000, 2001; Fu *et al.*, 2000; Han *et al.*, 2000, 2002; Wallwork & Sedgley, 2000). The advantageous features of the *T. fournieri* embryo sac make it easier – relative to many other angiosperms – to isolate vigorous egg cells. Mól (1986), for example, isolated egg cells of *T. fournieri* using enzymatic digestion. Imre & Kristóf (1999) also isolated protoplasts of embryo sac cells including egg cells using 2% cellulase Onozuka-R10 and macerozyme R10. Based on the successful isolation of nearly mature sperm from the pollen tube of *T. fournieri* (Chen *et al.*, 2006), we present a protocol for isolating mature, living egg cells and zygotes of *T. fournieri* using micromanipulation or a combination method of enzyme maceration and micromanipulation. Considering sperm cell of *Plumbago zeylanica* (Zhang & Russell, 1999) and *Nicotiana tabacum* (Yang *et al.*, 2005) displaying the velocity in a current field we measured the velocity of the egg cell and zygote and compared the mobility of both cells using electrophoresis.

Materials and methods

Torenia fournieri L. plants were grown under a 14 h: 10 h, light : dark cycle at 22 °C (dark) and 28 °C (light) at Xiamen University. Flower buds were emasculated before anthesis. The emasculated flowers were collected just at anthesis; 1 or 2 days after anthesis for egg cell isolation. The artificially pollinated flowers were collected at about 14 h and 18 h for zygote isolation.

Several ovaries were dissected under a stereomicroscope and the ovules were removed from the placenta by gentle scraping. All of the ovules were put into 2 ml of an isolation solution containing 5 mM $CaCl_2$, 5 mM KH_2PO_4, 0.7 mM $MgSO_4$, 3 mM MES (2-*N*-morpholino ethanesulfonic acid) and 3–9% (w/v) mannitol (197–500 mOsmol/kg H_2O) at a pH of 5.8. For mechanical isolation of an egg cell, the embryo sacs were dissected with an ultra-fine needle under an inverted microscope. The partial protrusion of the embryo sac through the micropyle of ovule facilitated penetration into the embryo sac wall and incision near the egg cell. The egg cell was then released by pressing the micropylar end of the embryo sac. Two synergid cells could be released together with the egg.

For isolation of egg cell and zygote using an enzyme, 0.1–0.2% cellulase (Onozyka R-10) and 0.1–0.2% pectinase (Serva) were added into the isolation solution. The ovules were incubated in the solution for 0.5 h with gentle shaking (30 rpm) and then dissected using a needle.

Micro-electrophoresis was conducted in a 6-cm Petri dish. Two small pieces of glass (cut from microscope slides) were attached with a glue to form a 5 mm wide, 5 cm long trough in the centre of the dish. Both ends of the trough were sealed with 2% agarose to form two 'reservoirs' on either side of the trough. The electrophoresis trough and the reservoirs were filled with low ionic buffer (van Oss & Fike, 1979), containing 10 mM NaH_2PO_4, 0.3 mM citric acid and 7% mannitol. Buffers with different pH (4–9) were tested to find the optimal pH. Two electrodes were separately immersed into the 'reservoirs'. Egg cells or zygotes isolated from the embryo sac were transferred with a microinjector into the trough in the centre of the optical field. Selected voltages were applied with two electrodes with constant current and the mobility of egg or zygote was observed using an inverted phase-contrast microscope. Velocity was measured using the recorded images and electrophoretic mobility was calculated by the equation (van Oss & Fike, 1979):

$$\mu = (d/t)/(V/day)$$

where μ is the electrophoresis mobility, d is the distance (in micrometres) travelled by cells during measurement, t is the time (in seconds) required by cells to travel that distance, V is the potential applied to the electrodes (in volts) and day is the length of the trough (in centimetres) (Zhang & Russell, 1999).

Results

Isolation of living egg cells

The stigma of *T. fournieri* consists of two halves or slivers, which are closed just when the petal opens. The two halves of the stigma usually open the next day after anthesis and are receptive to pollination for 2 days following anthesis. An egg and two synergids can be observed clearly at anthesis in the partially protruding embryo sac. The day after anthesis, a second nucleus in the central cell moves to the side of egg cell, developing to a physiological mature state for fertilization. Egg and synergid cells in the embryo sac are pear-like. The egg cell is bigger than both synergids and has several large vacuoles, which makes it easy to identify and separate from the two synergids and also from the nuclear cells (Fig. 1).

The protruded embryo sac was first excided in its central part using a glass needle and then the micropylar part of embryo sac was gently extruded; the egg and synergid cells spilled out from the cut end of embryo sac. Just released egg and synergid

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Table 1 The surface, size and mobility of *Torenia fournieri* egg cells and zygotes

	Area (μm²)	Volume (μm³)	Electrophoretic mobility (μm/s)
Egg	2590.87 ± 328.16	12473.45 ± 2343.20	1.078 ± 0.020
Zygote	2089.93 ± 423.24	9115.49 ± 2798.54	1.379 ± 0.023

Table 2 Effect of different osmolalities of isolation media on the isolation and conservation of egg cells at different stages

	Concentration of mannitol			
Stages	3% (197 mOsmol/kg)	5% (298 mOsmol/kg)	7% (399 mOsmol/kg)	9% (500 mOsmol/kg)
Anthesis	Ruptured	Could be isolated in good state	Difficult to isolate	Shrunken
1d after anthesis	Ruptured	Could be isolated in good state	Could be isolated	Shrunken
2d after anthesis	Ruptured	Difficult to isolate	Could be isolated	Shrunken

Table 3 Concentration of enzymes and mannitol in the maceration medium (%) and their relationship to egg and zygote isolation (2 d after anthesis)

		Concentration of mannitol	
Cellulase (%) (Onozyka R-10)	Pectinase (%) (Serva)	5% (298 mOsmol/kg)	7% (399 mOsmol/kg)
0	0	Could be isolated	Could be isolated (isolation rate 5%/2h)
0.1	0.1	Could be isolated	Easy isolated (isolation rate 18%/2h)
0.2	0.2	Ruptured	Easy isolated (isolation rate 18%/2h) but metamorphosed and ruptured

cells were still connected by some cytoplasm material (Figs. 2, 3), but soon became separated each other following the cytoplasm dissolving (Figs. 4, 5). Several large vacuoles occupied much of the egg cell volume, with the nucleus and most of the cytoplasm being located along the cell periphery (Fig. 4). The average surface area and volume of egg cells are 2590.87 ± 328.16 μm² and 12473.45 ± 234.20 μm³, respectively (Table 1). Mechanical isolated living egg cells are especially effective for the recognition of male and female gametes in non-enzyme *in vitro* fertilization assays.

Egg cells in embryo sacs at different stages displayed various sensitivities to the osmolarity of the isolation media (Table 2). The egg cells in embryo sacs of all stages were ruptured or shrunken in an isolation solution containing either 3% and 9% mannitol, indicating the osmotic pressures of these isolation media were too low or high. The egg cells in the flowers at anthesis and 1 day after anthesis could easily be isolated in a 5% mannitol isolation solution but it became difficult to isolate eggs cells that were collected 3 days after anthesis in the 5% solution. In the 7% mannitol solution, isolation was difficult with at-anthesis egg cells but easier with 1 day postanthesis egg cells. The isolated egg cells, however, from the 7% mannitol solution would not ‘keep’ as long as those obtained in the 5% mannitol. The isolation solution, therefore, containing 5% mannitol was judged to be the most suitable for egg cell isolation.

Our studies demonstrated that enzymes could evidently increase the egg cell isolation rate from 5% (2–3 egg cells in 50 intact ovules) to 18% (8–10 egg cells in 50 intact ovules) when 0.1% cellulase and 0.1% pectinase were added to the isolation solution (Table 3). When the enzyme levels were increased to 0.2%, the released egg cells were more difficult to identify because the ovules also released numerous protoplasts of various sizes. In addition, in 0.2% enzymes egg cells easily became anamorphic, suggesting a deleterious effect from the enzymes at the higher concentration. Isolated living egg cells can be combined into a pure population using a micromanipulator (Fig. 6), which is especially effective

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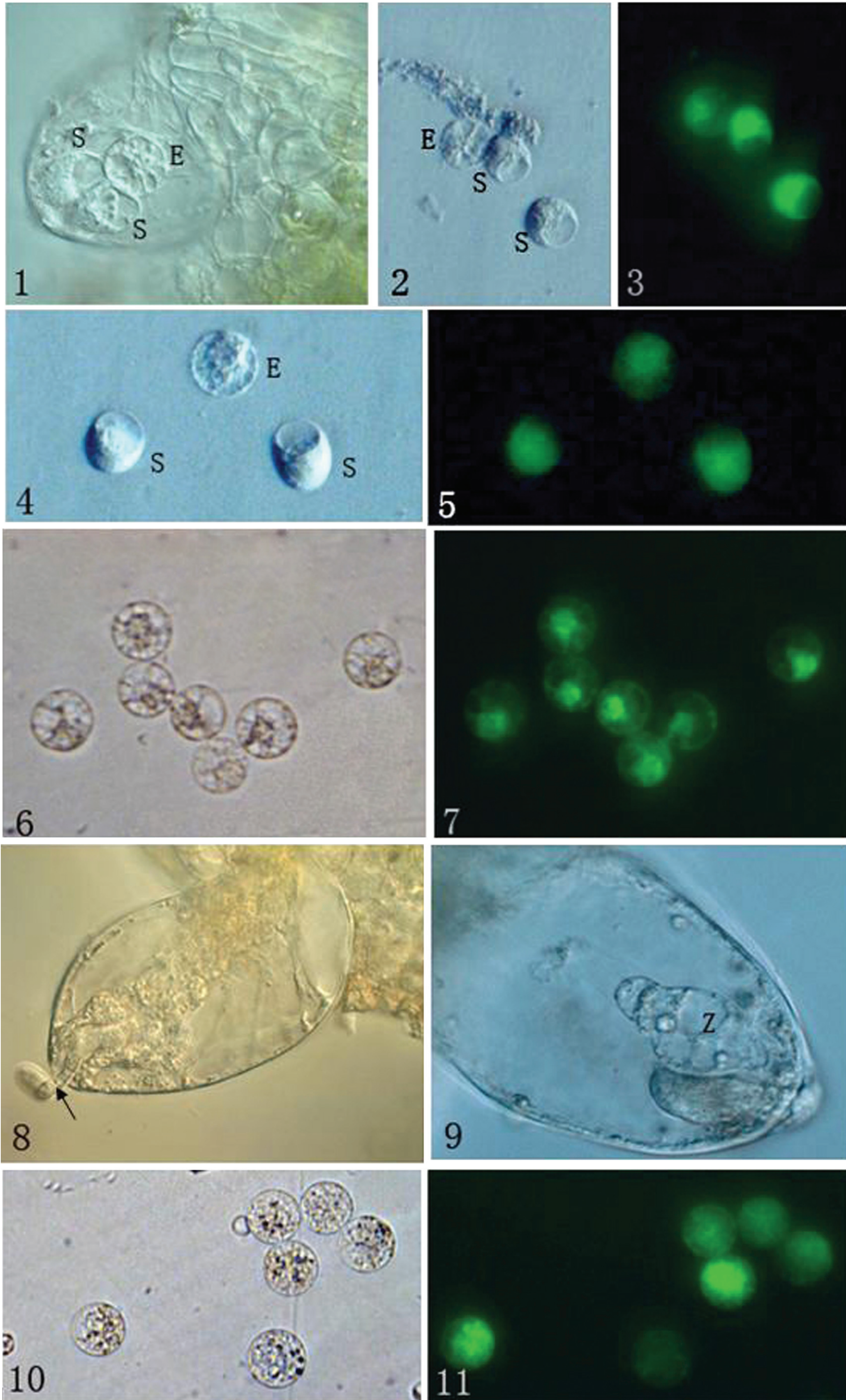

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for making a cDNA library of egg cell. The viability of isolated egg cells can apparently be determined by the presence of cytoplasmic fluorescence in the fluorescein diacetate (FDA) test at 4 h after isolation (Fig. 7).

Isolation of living zygotes

Most ovules displayed a pollen tube in its micropyle at 14 h after pollination and one synergid that could not be seen clearly (Fig. 8), suggesting that fertilization of the egg cell occurred at this time. Eighteen hours after pollination, the fertilized egg cell began to elongate in the embryo sac. The nucleus with a larger nucleolus migrated to the chalazal end of the cell, resulting in polarity of the cell (Fig. 9).

Isolation of the zygote occurred in the same manner as the above-mentioned egg-cell isolation method. Most zygote isolations were completed using cellulase and pectinase because the enzymes substantially improved this process. In the 7% mannitol solution, 2–4 zygotes could be isolated from 50 ovules (about 5%). when 0.1% cellulose and 0.1% pectinase were added to the isolation solution, however, the number of isolated zygotes could exceed 10 (>20%) in 50 ovules. Zygotes took on a spherical shape at 0.5 h after the ovule was incubated in an enzymatic maceration mixture. When the zygote was released from the embryo sac and assumed a round shape, its nucleus was located in the center of the cell with more cytoplasm surrounding nucleus (Fig. 10). The average surface area of zygotes was 2089.93 ± 423.24 μm^2 and their volume 9115.49 ± 279.54 μm^3 (Table 1), which was smaller than that of egg cell. The isolated zygotes could be collected into a pure population using a micromanipulator (Fig. 8). In the viability test with FDA, zygote fluorescence remained viable for 5 h in the 7% mannitol solution and was mainly located in its central region, which was different from the egg cell (Fig. 11).

Compared with egg isolation, zygote isolation was most effectively completed in a solution of higher osmolality. While zygotes could be isolated in a 5% mannitol solution, it was necessary to press the micropylar end to release them when the embryo sac was excided by a needle. In addition, zygotes swelled soon after excision and were easily broken after only 2 h, as opposed to 5 h of viability in the 7% mannitol solution.

Micro-electrophoresis of egg and zygote

Buffer pH can have a major effect on the viability and electrophoresis of both egg cell and zygote. The movement of egg cell or zygote in an electric field depends on its surface charge. The pH of the buffer can alter egg cell and zygote mobility and can even poison both cells. In either pH 4 or 9 buffers, both egg cell and zygote became brown and died immediately upon release into the buffer. In the pH 5 or 8 buffers, egg cells and zygotes all migrated toward the negative pole at various speeds; both cells also broke shortly after voltage was applied to the electrophoretic solution. In the pH 6 buffer, both egg and zygote migrated toward the negative pole, moved faster than in the pH 7 buffer and remained intact for 40 min during electrophoresis. The optimal buffer pH for electrophoresis of egg cells and zygotes in *T. fournieri*, therefore, is 6 (Table 4).

When egg cell or zygote was placed separately into an electric field (voltage 70 mV, current 10 mA) filled with the optimal buffer, they both migrated toward the negative pole, indicating positive charges on the surface of the cells (Figs. 12, 13). The egg cell moved slower than zygote. The average velocity of 10 egg cells was 1.078 ± 0.020 μm/s, while the average velocity of 25 zygotes was 1.379 ± 0.023 μm/s (Table 1), suggesting that zygotes may have a larger surface charge.

Figure 1 In an ovule, naked embryo sac partly protruding out micropylar and an egg cell (E) and two synergids (S) could be seen clearly. Scale ×400.

Figure 2 Just released one egg cell (E) and two synergid cells (S). Scale ×400.

Figure 3 The viability of isolated egg cell and synergids was shown by FDA reaction. Scale ×400.

Figure 4 Egg cell (E) is different with two synergids (S). Scale ×400.

Figure 5 The viability of three cells was evaluated by FDA reaction. Scale ×400.

Figure 6 Seven collected egg cells after 4 h isolated. Scale ×400.

Figure 7 The viability of these egg cells was evaluated by FDA reaction. Scale ×400.

Figure 8 In an ovule 14 h after pollination, a pollen tube (arrow) residue could be seen. Scale ×400.

Figure 9 In an ovule 18 h after pollination, zygote began to elongate. Scale ×400.

Figure 10 Six collected zygotes. Scale ×400.

Figure 11 The viability of these zygotes was shown by FDA reaction. Scale ×400.

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Table 4 pH of buffer effect on egg cells and zygotes during both electrophoresis

pH	Living state	Surface charge	Movement to negative pole
4	Immediately broken		
5	Broken after in current field	+	–
6	Move rapidly; none broken[a]	+	–
7	Move slower than in pH 6	+	–
8	Broken after in current field	+	–
9	Immediately broken		

[a]The best condition.

Discussion

At present, the biggest obstacle to using *in vitro* fertilization to perform research on reproduction in higher plants is the isolation of male and female gametes, especially the isolation of the egg cell. Egg cells were isolated using two methods, either mechanical micromanipulation or a combination of enzyme maceration and micromanipulation. These two methods have both advantages and disadvantages. Mechanical isolation of an egg cell was more difficult and fewer cells were isolated, but this method avoided a potential problem with enzyme digestion of the proteins located on the surface of egg plasma membrane (Cao & Russell, 1997). In this assay, the egg cells of *T. fournieri* can be isolated without enzymatic digestion, although the number recovered was only 4–6% (2–3/50 ovules). The isolated egg cells are comparatively similar to those *in vivo*, so that the results achieved using isolated cells are appropriate for defining fertilization mechanisms, especially in male and female gametic recognition studies. Enzymes can poison the isolated cell and digest some membrane proteins that may interfere with the recognition of male and female gametes (Leduc *et al.*, 1995). In this assay, about 18% of the egg cells (8–10/50 ovules) could be isolated in a 0.1% cellulase and 0.1% pectinase maceration solution. These isolated egg cells were followed by subsequent preparation of cDNA libraries in order to separate egg genes. The successful isolation of the male and female gametes provided the opportunity to study zygote formation outside the female gametophyte or embryo sac.

Isolation of the zygote is also an important new field in the study of plant developmental biology (Kumlehn *et al.*, 1999). With the successful isolation of egg cells and zygotes, comparisons of cDNA libraries between egg and zygote can be used to isolate specific genes of the egg cell and zygote, which may shed light on the mechanisms of egg activation. Dresselhaus *et al.* (1996) first constructed two cDNA libraries from 128 maize egg cells and another library from 104 maize zygotes generated by *in vitro* fertilization (18 h after *in vitro* fertilization), to isolate newly expressed genes in zygotes or genes expressed more abundantly after fertilization. Plant zygotes exhibit a high degree

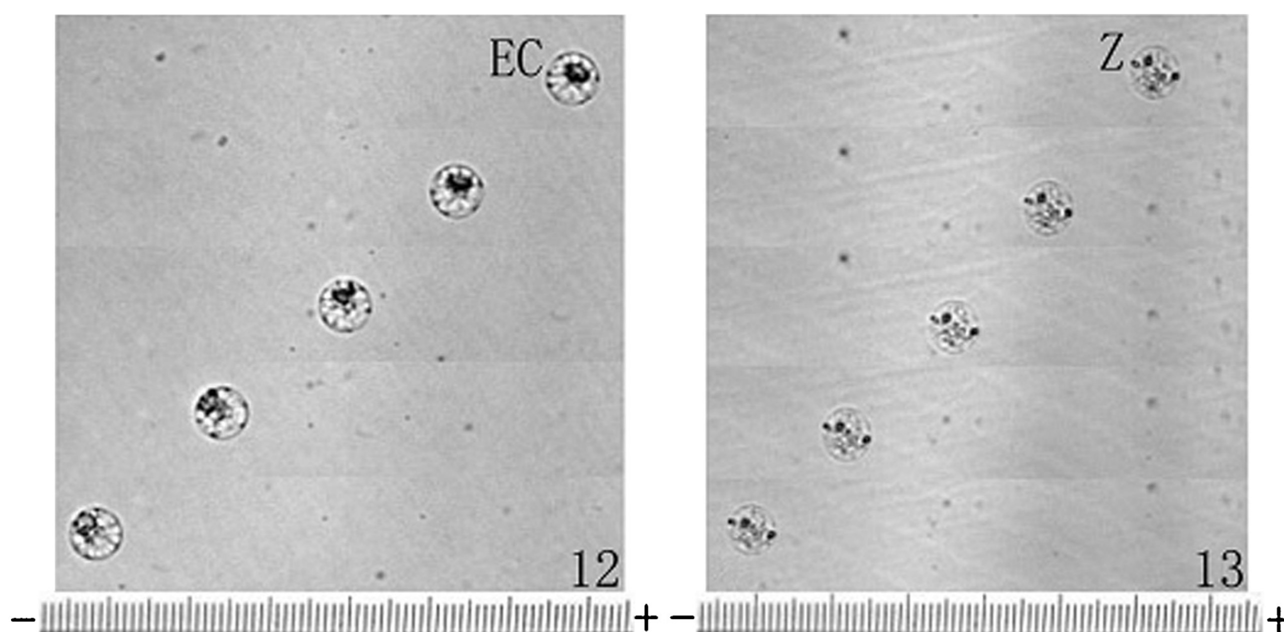

Figure 12 A egg cell was moving toward the negative pole in an electric field. The time interval was 4 s. Scale ×200.

Figure 13 A zygote was moving toward the negative pole in an electric field. The time interval was 4 s. Scale ×200.

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of vigor *in vivo* and a higher rate of cell division than somatic cells. The isolated *in vitro* and *in vivo* zygotes divided easily and essentially underwent embryogenesis to produce fertile plants when cultured (Kranz & Lörz, 1993; Holm *et al.*, 1994; Leduc *et al.*, 1996; Kumlehn *et al.*, 1997, 1998). Then, successful isolation and culture of zygotes provides the opportunity for using zygotes as recipient cells in the transformation of higher plants, which may improve transformation frequency (Wang *et al.*, 2006).

The electrophoretic analysis of isolated egg cells and zygotes of *T. fournieri* indicated that the surfaces of both were positively charged, with an average electrophoretic mobility of $1.078 \pm 0.020\,\mu m/s$ and $1.379 \pm 0.023\,\mu m/s$, respectively. The significance, if any, of the surface charge difference between egg cell and zygote is not clear. The more rapid movement of the fertilized egg cell (zygote) in an electric field under the conditions of this study is probably caused by the higher surface charge. Whether this surface charge increase was associated directly with the sperm, some other influence or a combination of factors, is unknown. Further research to explore the cause and consequence of this alteration in charge may further elucidate the intricate reproductive mechanisms of higher plants.

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