Egg cell isolation in *Datura stramonium* (Solanaceae)

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Viable egg cells of *Datura stramonium* (Solanaceae) were isolated using enzymatic digestion and mechanical dissection. The three cells comprising the egg apparatus (the egg cell and two synergids) were removed from the female gametophyte by altering the micropylar position with a dissection needle. Generally, the egg apparatus cells were readily released from the cut end of the ovule. In order to isolate the egg cells, ovules were placed in an isolating solution containing 0.04% $CaCl_2$, 1% BSA, and 12% mannitol, seven egg apparati were isolated from 40 ovules within one hour period. However, in some cases, it was difficult to separate the egg cells from the two synergids. Separation of the egg and synergid cells required incubating the ovules in an enzymatic solution containing 1% pectinase (Serva), 1% cellulase (Onozuka RS), and 12% mannitol for 30 min, and transferring them to the above-mentioned isolating solution. Following this protocol, eight egg cells were isolated from 40 ovules within two hours, and the egg cells were easily separated from the two synergids. Subsequently, isolated egg cells were used for *in vitro* fertilization to explore the mechanisms of egg development and fertilization.

Introduction

The isolation and subsequent *in vitro* fertilization of sperm and egg cells under controlled conditions provides a useful means to study higher plant fertilization in individual cells in the absence of other somatic tissue. In this way, the mechanisms controlling fertilization, male and female gamete recognition, and egg cell activation can be investigated. Isolated egg cells are not only used for *in vitro* fertilization, but also provide an experimental basis to evaluate egg and zygote development. Hu *et al*. (1985) isolated viable egg cells from tobacco. Kranze *et al*. (1993) conducted *in vitro* fertilization of maize using isolated sperm and egg cells, and generated fertile plants. How-

ever, the second *in vitro* fertilization of higher plants was not reported until 14 years later in rice (Uchimi *et al*. 2007). The time span between these papers suggests that the establishment of *in-vitro* fertilization in higher plants is challenging, and studies have shown that the major obstacle is egg-cell isolation. To date, the number of viable egg cells successfully isolated has been limited to approximately 10 species, which has restricted progress in the *in-vitro* fertilization research (Wang *et al*. 2006). *Datura stramonium* has been used as a Chinese medicine for a long time. In the present study, we introduced a protocol for successful isolation of mature, living egg cells from *D. stramonium* using a method combining enzyme maceration and micromanipulation.

Material and methods

Plants of *Datura stramonium* (Solanaceae) were grown at the Xaimen Botany Garden. In the Xaimen area, the species blooms in April, and its flowering period is approximately one month. The 5-merous corolla drops within 7 days after anthesis. The flower has a bicarpellate ovary with two locules. Approximately 300 anatropous ovules attach on the axile placenta. Ovules were collected 1, 3, 5 and 7 days after anthesis and incubated in an enzymatic solution containing 0%–1.5% pectinase (Serva), 0%–1.5% cellulase (Onozuka R-10), and 8%–15% mannitol for 30 min at 25–27 °C with gentle shaking. The ovules were removed from the enzymatic solution, and subsequently washed twice with a solution containing 0.04% $CaCl_2$, 1% bovine albumin (Amresco), 8%–15% mannitol, and dissected using an inverted microscope. The outline of the female gamete in *D. stramonium* is apparent and its egg apparatus is clearly evident after enzymatic maceration, which indicates the precise position for ovule dissection (Fig. 1A). A glass micro-needle was used to cut through the middle of the ovules. A gentle pushing on the micropylar region releases the egg-apparatus cells from the cut end (Fig. 1B). The isolated egg cells were collected using a micromanipulator. Cell viability was evaluated using a fluoresencein diacatate (FDA) reaction.

Results

Isolation of the egg apparatus at different developmental stages

The egg apparatus was easily distinguished in the ovule as being composed of an egg cell and two synergids, because the three cells are connected and larger than the nucellus cells. After isolation, the egg and two synergid cells were distinguished by their cell structure and morphology. The synergid cell nuclei were located on one side of each cell and display the same polarity, which was unlike that of the egg cell (Fig. 1C). After recovery of the egg apparatus, the viability of the isolated cells was evaluated using the FDA reaction. The three isolated cells were strongly fluorescent, which suggests that the cells remained viable also after the isolation procedures (Fig. 1D). The style was about 30 cm long, and the corolla became senescent by the 7th day after anthesis. When the ovules were dissected 7 days after anthesis, the egg apparatus was released from the ovules, suggesting that fertilization had not occurred. Developmental differences were observed among the three apparatus cells isolated at different ovule developmental stages.

Three days after anthesis, the three cells were nearly the same size. Five days after anthesis, the two synergid cells were morphologically different. One became round and the other remained pyriform. One hour after isolation, the FDA reaction revealed well developed fluorescence in the egg and pyriform synergid cells, indicating that viability was maintained 1 h after the procedure. Three hours after isolation, the egg cell lost fluorescence (Fig. 1E and F). In ovules 7 days after anthesis, the egg cell is larger than the two synergid cells, and synergids differ in size (Fig. 1G). One hour after isolation, the egg-apparatus cells remained fluorescent, but the smaller synergid was less fluorescent than the larger synergid cell (Fig. 1H). The three egg-apparatus cells were separated and collected individually using a micromanipulator to specifically isolate the egg cell (Fig. 1I). Some of the egg cells changed shape during separation, but rapidly recovered the characteristic roundness. The egg cells lacking a connection with the two synergid cells continued to fluoresce, indicating viability of the cells (Fig. 1J). The osmolality of the isolating solution affected the shape of the egg cells. Cells shrank in higher osmotic solutions (Fig. 1K) and swell in lower osmotic solutions (Fig. 1L and Table 1). The egg cells were collected into a group using a micromanipulator (Fig. 1M and N), and some egg cells remained fluorescent 2 hours after isolation (Fig. 1O).

Selection of optimal isolation conditions

Approximately 300 ovules are produced in each *D. stramonium* ovary. Typically, 40 ovules can be dissected in one hour. The enzymatic components in the digesting solution and the osmolality
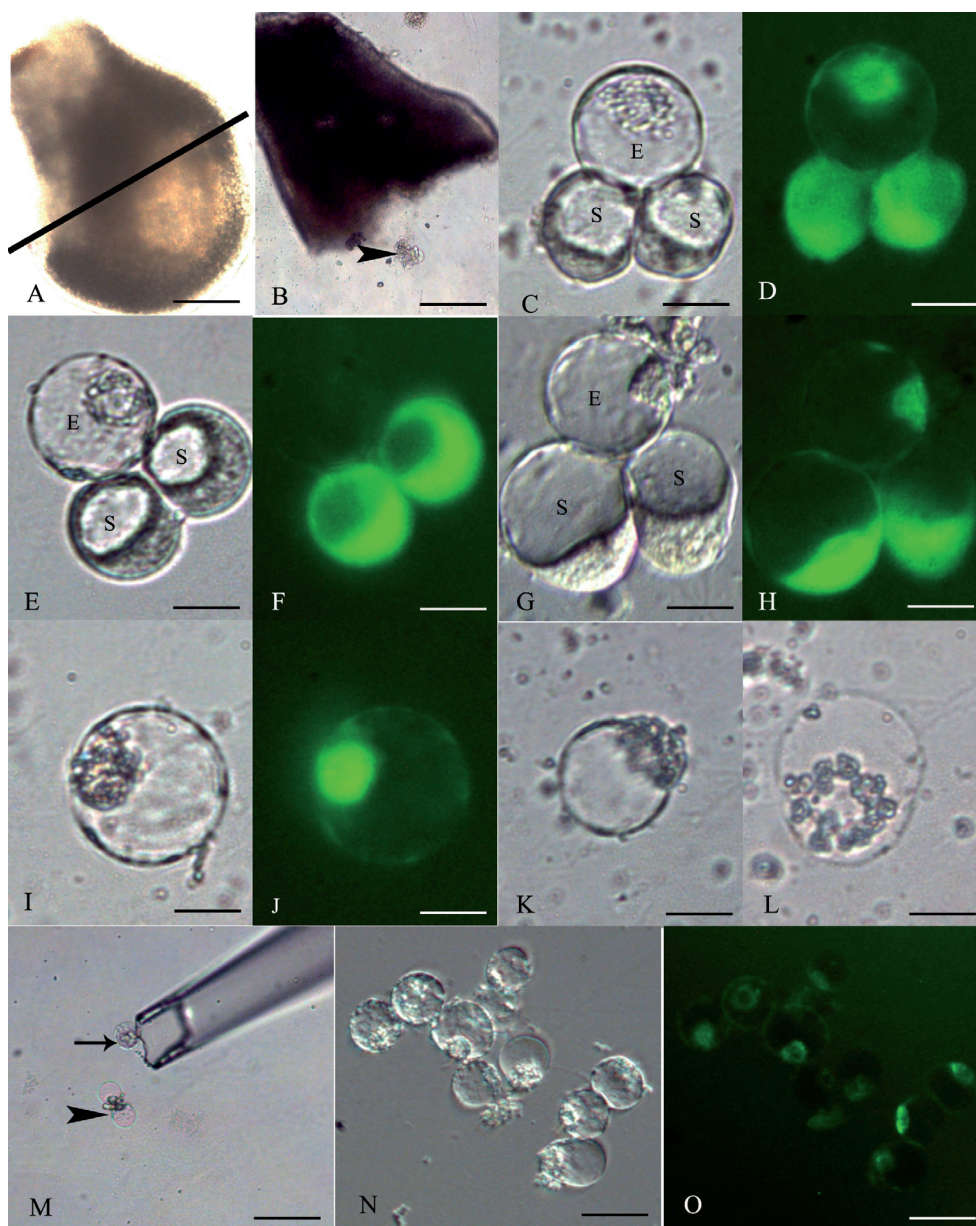
Fig. 1. Egg cell isolation in *Datura stramonium.* — **A**: An ovule showing the female gametophyte profile; the line indicates the dissection position (×120, bar = 100 *μ*m). — **B**: A half ovule showing an egg apparatus (arrow) released from the cut end of an ovule (×240, bar = 50 *μ*m). — **C**: Three cells of an egg apparatus isolated from an ovule; the two synergid cells exhibit the same polarity; S = synergid, E = egg cell (×1200, bar = 10 *μ*m). — **D**: Three cells of an egg apparatus showing viability after the FDA reaction (×1200, bar = 10 *μ*m). — **E**: Three cells of the egg apparatus isolated at anthesis; S = synergid, E = egg cell (×1200, bar = 10 *μ*m). — **F**: Three isolated cells at 3 h, two synergids still show fluorescence, but the egg cell has lost fluorescence (×1200, bar = 10 *μ*m). — **G**: Three cells of the egg apparatus isolated from an ovule 7 days after anthesis; one synergid is larger than the other; S = synergid, E = egg cell (×1200, bar = 10 *μ*m). — **H**: Egg apparatus cell viability after the FDA reaction (×1200, bar = 10 *μ*m). — **I**: An isolated egg cell (×1200, bar = 10 *μ*m). — **J**: Egg cell viability after the FDA reaction (×1200, bar = 10 *μ*m). — **K**: A shrinking egg cell in a higher osmotic solution (×1200, bar = 10 *μ*m). — **L**: A swelled egg cell in a lower osmotic solution (×1200, bar = 10 *μ*m). — **M**: Egg cell collection using a micromanipulator; arrow indicates egg cell and arrowhead two synergid cells (×480, bar = 25 *μ*m). — **N**: Ten collected egg cells (×800, bar = 15 *μ*m). — **O**: Some egg cells still show fluorescence 2 h after isolation (×800, bar = 15 *μ*m).

in the dissecting solution are effective in releasing the egg apparatus cells and maintaining the viability of the egg cells. Isolation of the egg apparatus was possible without digestion, but a longer time was necessary for dissection of each ovule, and resulted in releasing of only one or two egg apparati in one hour from 40 ovules. When the egg apparatus was isolated without enzymatic maceration, a small amount of cytoplasm from the central cell remained on the surface of the egg apparatus. This residual cytoplasm linked the three cells together, causing difficulty in separating the egg and two synergid cells. Furthermore, in attempts to separate the cells under these conditions, the egg cell was broken.

To avoid continued enzyme digestion that could reduce or eliminate egg viability, the ovules were incubated in the enzymatic solution containing 0.5%, 1.0%, or 1.5% cellulase and pectinase for 30 minutes. The enzymatic solution was then removed and the ovules were dissected in an enzyme-free solution. The most effective means to isolate egg cells was to treat the ovules with the enzymatic solution. The eggs were readily released from the ovules and the cell surface was clear. In addition, the egg cells were separated from the two synergids with little difficulty. The enzyme concentration had no obvious effect on the isolation frequency of egg cells, and seven to nine egg cells were recovered from each enzymatic treatment. However, egg cells isolated at the 0.5% enzyme concentration generally remained fluorescent for a longer time.

The osmolality of the isolating solution is another factor affecting egg-cell isolation. In 8% mannitol, the greatest numbers of egg cells were recovered, but they became increasingly inflated and the cytoplasm density decreased. The egg cells also exhibit a large central vacuole, and show the highest vacuolization of all cells in the

egg apparatus. The FDA reaction revealed that these egg cells lose viability already 30 minutes after isolation. In 15% mannitol, the egg cells were isolated, but began to shrink and lose fluorescence soon after isolation. In 10% and 12% mannitol, fewer egg cells were recovered than in 8% mannitol, but the cells remained fluorescent 2 hours after isolation (Table 1). The egg cells released in 10% mannitol, became round immediately. The egg cells released in 12% mannitol were elliptical, but later became round. Therefore, the osmolality of a 12% mannitol isolating solution may be near that of *in vivo* conditions.

Discussion

In higher plants, egg-cell isolation is the most challenging obstacle to overcome in *in-vitro* fertilization techniques. The isolation of egg cells can serve as an experimental basis to perform egg cell developmental research at the molecular level. In the present study, the egg cells of *D. stramonium* were isolated using mechanical micromanipulation or a combination of enzyme maceration and micromanipulation. Hence, two successful and reliable applications are available, but advantages and disadvantages are inherent in both protocols. For *in vitro* fertilization, realistically only one egg cell is required for each fertilization attempt. However, exploration of the development and recognition mechanisms of male and female gametes requires that a certain number of isolated egg cells must be isolated. Isolation techniques must consider a number of potential risks, the most important of which is avoidance of enzyme digestion of proteins located on the egg plasma membrane surface. Digestion could render the egg inviable. Therefore, although mechanical isolation of an egg cell was more difficult, and fewer cells were

Table 1. Effect of osmolality on egg cell isolation.

	8% mannitol 523 mOsmol/kg H_2O	10% mannitol 638 mOsmol/kg H_2O	12% mannitol 723 mOsmol/kg H_2O	15% mannitol 944 mOsmol/kg H_2O
Number of egg cells	10/40	8/40	7/40	5/40
Number of living egg cells	0	5	5	1
State of egg cells	inflating	normal	normal	shrinking

recovered, the egg cells would likely operate similarly to *in vivo* fertilization.

To date, egg cells of *Plumbago zeylanica* (Cao & Russell 1997) and *Torenia fournieri* (Chen *et al.* 2008) were isolated using mechanical isolation, but the most successful egg-cell isolations required enzyme digestion of the ovule somatic cells (Hoshino *et al.* 2006). However, the application of molecular biological methods to research egg-cell development requires a greater number of egg cells. Dresselhaus *et al.* (1994) constructed a cDNA library from 128 maize egg cells using RT-PCR, and for the first time isolated genes expressed in egg cells. They subsequently compared the egg cDNA library with a library of 104 maize zygotes, and isolated newly expressed zygote genes following *in vitro* fertilization (Dresselhaus *et al.* 1996). In the present study using enzyme maceration and micromanipulation, the number of isolated egg cells was increased to nine after one hour. These results demonstrate that an adequate number of egg cells can be isolated to study their development using molecular biological assays.

In angiosperms, synergids are considered pivotal in assuring successful fertilization. The synergid cells direct pollen-tube growth toward the female gametophyte, and facilitate the entrance of the tube into the female gametophyte. Once the pollen tube enters the synergid cell, its growth is arrested, the tip of the tube breaks, and two sperm cells are released. This sequence of events is also synergid dependent (Li *et al.* 2009). In mature female gametophytes of *Allium tuberosum* (Liliaceae), the two synergids display distinct size differences. However, that result was obtained by section, again resulting in a two-dimensional depiction, and additional lines of evidence have not confirmed these results (Tian & Yang 1991).

In the present study, the two isolated synergids also differed in size, which confirmed that size differences are the result of developmental progression. An interesting question is born out of this observation: Are the synergids related to pollen-tube reception, and if so, which of the two cells? In the female gametophytes of the *Polygonum* type, which bear two synergids, one synergid sometimes degenerates before the pollen tube arrives and serves as a receptive cell

for pollen tube entry. The other synergid degenerates, or persists but does not accept the pollen tube. Therefore, the question is raised: Does a pollen tube enter a synergid by chance or by selection? Unfortunately, few reports regarding synergid development and even fewer on structural and functional differences between two the synergid cells are available.

Acknowledgements

References

Cao, Y. J. & Russell, S. D. 1997: Mechanical isolation and ultrastructural characterization of viable egg cells in *Plumbago zeylanica*. — *Sexual Plant Reproduction* 10: 368–373.

Chen, S. H., Yang, Y. H., Liao, J. P., Kuang, A. X. & Tian, H. Q. 2008: Isolation of egg cells and zygotes of *Torenia fournieri* L. and dtermination of their surface charge. — *Zygote* 16: 179–186.

Dresselhaus, T., Lörz, H. & Kranz, E. 1994: Representative cDNA libraries from few plant cells. — *Plant Journal* 5: 605–610.

Dresselhaus, T., Hagel, C., Lörz, H. & Kranz, E. 1996: Isolation of a full-length cDNA encoding calreticulin from a PCR library of *in vitro* zygotes of maize. — *Plant Molecular Biology* 31: 23–31.

Hoshino, Y., Murata, N. & Shinoda, K. 2006: Isolation of individual egg cells and zygotes in *Alstroemeria* followed by manual selection with a microcapillary-connected micropump. — *Annual Botany* 97: 1139–1144.

Hu, S. Y., Li, L. G. & Zhu, C. 1985: Isolation of viable embryo sacs and their protoplasts of *Nicotiana tabacum*. — *Acta Botanica Sinica* 27: 337–344.

Kranz, E., Bautor, J. & Lörz, H. 1991: *In vitro* fertilization of single, isolated gametes of maize mediated by electrofusion. — *Sexual Plant Reproduction* 4: 12–16.

Kranz, E. & Lörz, H. 1993: *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. — *Plant Cell* 5: 739–746.

Li, D. X., Lin, M. Z., Wang, Y. Y. & Tian, H. Q. 2009: Synergid: a key link in fertilization of angiosperms. — *Biologia Plantarum* 53: 401–407.

Tian, H. Q. & Yang, H. Y. 1991: Embryo sac development and embryogeny in *Allium tuberosum*. — *Journal Wuhan Botany Research* 9: 5–10.

Uchiumi, T., Uemura, I. & Okamoto, T. 2007: Establishment of an in vitro fertilization system in rice (*Oryza sativa* L.). — *Planta* 226: 581–589.

Wang, Y. Y., Kuang, A., Russell, S. D. & Tian, H. Q. 2006: In vitro fertilization as a tool for investigating sexual reproduction of angiosperms. — *Sexual Plant Reproduction* 19: 103–115.