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Isolation of eggs and synergids in *Ceiba speciosa*

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Viable egg and synergid cells of *Ceiba speciosa* were isolated using enzymatic digestion and mechanical dissection. The ovules were digested by 1% (w/v) cellulase RS, 0.3%~0.8% (w/v) pectolyse Y-23, and 0.8% (w/v) hemicellulase for 40–50 min and then transferred for dissection to an isolating solution containing 9% mannitol without enzymes. The two integuments of the ovules were peeled off and the nucellus was cut transversely through the middle. The three cells comprising the egg apparatus (the egg cell and two synergids) were released from the excised nucellus by pushing the micropylar end with a dissecting needle. Dissection of 50 ovules in the isolating solution yielded 19 groupings of egg apparatus cells within an hour. Following this protocol, egg and synergid cells of *C. speciosa* can be effectively isolated.

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 the absence of other somatic tissue. In this way, the mechanisms controlling fertilization, male and female gamete recognition, and egg cell activation can be directly investigated. Isolated male and female gametes are not only used for *in vitro* fertilization, but also provide an experimental basis to evaluate sperm, egg and zygote development, such as creating egg cDNA libraries (Dresselhaus *et al*. 1996, Le *et al*. 2005) and isolating special sperm gene expression (Singh *et al*. 2008, Gou *et al*. 2009). Hu *et al*. (1985) isolated viable egg cells from tobacco. Kranz *et al*. (1991) and Kranz and Lörz (1993) conducted *in vitro* fertilization of maize using isolated sperm and egg cells, and generated fertile plants. However, the next *in vitro* fertilization of higher plants was not reported until 14 years later in rice (Uchimi *et al*. 2007). This time lapse suggests that *in vitro* fertilization methods in higher plants are challenging, and studies have shown that the major obstacles are the isolation of the egg cell and the cell cycle congruity. To date, viable egg cells have been isolated successfully in approximately ten species, and there has been restricted progress in *in vitro* fertilization research (Wang *et al*. 2006). In addition, all reports of successful egg isolation are for herbaceous plants. In the present study, we developed a protocol for isolating living egg

and synergid cells from *Ceiba speciosa*, a woody plant, using a method combining enzyme maceration and micromanipulation.

Material and methods

Ceiba speciosa is a deciduous tree native to the tropical and subtropical forests of South America. The investigation reported here was carried out on plants grown on the campus of Xaimen University. At the Xaimen study site, this species flowers during October (Fig. 1A and B), and the duration of flowering is approximately one month. The species is pentacarpellate and bears a pentamerous ovary with over 100 anatropous, bitegmic, crassinucellate ovules (Fig. 1C). The antipodal cells degenerate at anthesis and only one egg cell, two synergids and a central cell remain in the female gametophyte. When the flowers reached full anthesis, the ovules were collected from ovaries and incubated in an enzymatic solution containing 1% (w/v) cellulase RS (Yakult Pharma Ceuticalind Co. LTL, Tokyo, Japan), 0.3%~0.8% (w/v) pectolyse Y-23 (Kikkoman Corp., Tokyo, Japan), 0.8% (w/v) hemicellulase (Sigma-Aldrich Chemical Co., St. Louis, MO), 0.04% (w/v) $CaCl_2$, 1% (w/v) bovine serum albumin (BSA), and 6%–11% (w/v) mannitol for 40–50 min at 25–27 °C with gentle shaking. The enzymatic solution was removed, and the ovules were washed twice in the non-enzymatic portion of the above-mentioned solution and then dissected using an inverted microscope. The isolated egg and synergid cells were collected using a Leica mechanical micromanipulator. Cell viability was evaluated by staining with fluorescein diacetate (FDA, Sigma-Aldrich) (Heslop-Harrison & Heslop-Harrison 1970).

Results

Isolation of egg and synergid cells

The female gametophyte of *C. speciosa* is embedded deeply in the crassinucellate ovule, and cannot be seen. Therefore, the two integuments must first be removed. However, in the washing solution containing 0.4% (w/v) $CaCl_2$, 1% (w/v) BSA, 8%–15% (w/v) mannitol but no enzyme, the ovule was difficult to dissect further. Without enzymatic digestion, dissection of each ovule is a slow and tedious process that results in the isolation of only one or two egg apparatus grouping of cells in 1 hr from 50 ovules. Furthermore, without enzymatic treatment, attempts to separate the cells of the egg apparatus usually result in damage to the egg cell.

With the addition of enzymes to the digestion solution, the ovules were relatively easy to dissect, and the egg apparatus was also easily released. Following 40 min incubation in the enzymatic solution, the two integuments were especially easy to remove from the ovule. The nucellus without surrounding integuments displayed an outline of the egg apparatus and central cell of the female gametophyte, which provided a precise guide for its dissection (Fig. 1D). Then, the nucellus was transversely cut into two parts (Fig. 1E). The egg and synergids are larger than the nucellar somatic cells and easily distinguishable from them (Fig. 1F). An egg apparatus was then released from the cut end by pushing the micropylar end of the nucellus (Fig. 1G). The cells of the released egg apparatus are easy to distinguish in the solution by their structure and morphology. The two synergid cells are of similar polarity, contain denser cytoplasm than the egg, and their nuclei are located on the micropylar pole of the cell while the nucleus of the egg cell resides at the chalazal pole (Fig. 1H). The three isolated cells displayed fluorescence, which suggested that they remained viable after isolation (Fig. 1I). The egg cell, separated from the two synergids (Fig. J), showed well-developed fluorescence (Fig. 1K). The isolated egg cells could be easily collected one by one with a micromanipulator (Fig. 1L and M). These egg cells maintained strong fluorescence for over 1 h (Fig. 1N), which disappeared 2 h after isolation. Using this isolation procedure, 8–15 egg cells could be collected from 50 ovules within 1 h (Fig. 1O). Two synergid cells with identical polarity and dense cytoplasm (Fig. 1P) were collected at full anthesis using the same method (Fig. 1Q). These cells also displayed fluorescence for 1 h (Fig. 1R). The isolated synergid cells could be collected one by one (Fig. 1S).

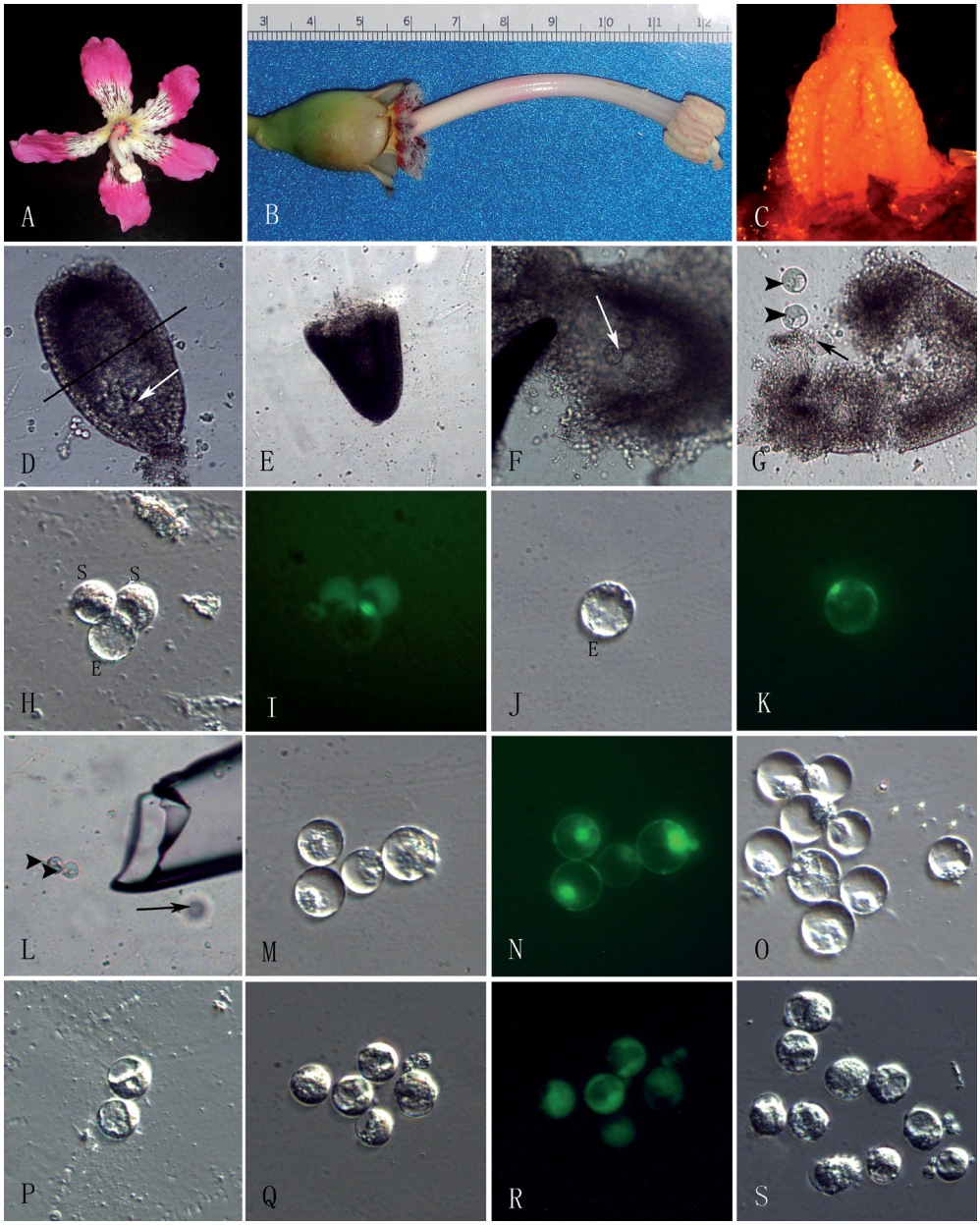

Fig. 1. Isolation of eggs and synergids in *Ceiba speciosa*. — **A**: A flower of *C. speciosa*. — **B**: A gynoecium of *C. speciosa*. — **C**: Ovules in an ovary after peeling off the ovary wall. — **D**: A nucella after two integuments were peeled off; the dark line represents cut position and the arrow shows the egg apparatus; bar = 100 μm. — **E**: Half of an ovule cut transversely; bar = 100 μm. — **F**: Egg apparatus cells (arrow) becoming released from the cut ovule face by pushing on the micropyle; bar = 50 μm. — **G**: Two synergid cells (arrowheads) and an egg cell (arrow) released from the cut ovule by pushing on the micropyle; bar = 50 μm. — **H**: Three egg apparatus cells isolated; S: synergids; E: egg cell; bar = 10 μm. — **I**: Same three cells as in H displaying viability after staining with FDA; bar = 10 μm. — **J**: An isolated egg cell; bar = 10 μm. — **K**: Same egg cell as in J displaying viability after staining with FDA; bar = 10 μm. — **L**: Two synergid cells (arrowheads) and egg cell (arrow) being collected with a micromanipulator; bar = 40 μm. — **M**: Four collected egg cells; bar = 10 μm. — **N**: Same four egg cells as in M displaying viability after staining with FDA; bar = 10 μm. — **O**: Nine egg cells collected after 1 h from starting the procedure; bar = 10 μm. — **P**: A pair of synergid cells; bar = 10 μm. — **Q**: Five isolated synergid cells; bar = 10 μm. — **R**: Same five synergid cells as in Q displaying viability after staining with FDA; bar = 10 μm. — **S**: Five pairs of isolated synergid cells; bar = 10 μm.

Selection of optimal isolation conditions

Generally, about 50 ovules can be dissected in 1 h. The enzymatic components in the digesting solution and the osmolality in the dissecting solution were effective for dissecting ovules, releasing the egg apparatus cells, and maintaining the viability of the egg cells. Among the enzymes, pectinase (pectolyse Y-23) was mainly effective for digesting the integument and softening the nucellar cells. Although the nucellus could be easily dissected at a higher concentration of pectolyse Y-23 (0.8%), the egg apparatus cells adhered, and it was difficult to separate the egg from the two synergids. In this case, 33% (15/50) of the egg cells could be isolated. Pectolyse Y-23 at 0.3% was effective for isolating 38% (19/50) the egg apparati (Table 1). The role of cellulase and hemicellulase in the digesting solution was mainly to digest the cell walls of the egg apparatus so as to release their protoplasts. In this condition, the eggs could be separated from the synergids with little difficulty.

The osmolality (determined from the concentration of mannitol) of the enzymatic digesting and dissecting solutions was yet another factor affecting egg cell isolation and maintaining the viability of the egg cells. In 6% mannitol, only 12% (6/50) egg cells could be dissected, and the cells became increasingly inflated with reduced cytoplasm density. Staining with FDA indicated that these egg cells lost viability 30 minutes after isolation. In 12% mannitol, 18% (9/50) egg cells could be dissected. However, the cells began to shrink and lost fluorescence soon after isolation. With 9% mannitol in digesting and dissecting solutions and 36% (18) egg cells could be isolated from 50 ovules in 1 h, and the cells maintained fluorescence (equivalent to viability) for up to 1 h after isolation (Table 2).

Discussion

The isolation of egg cells can provide a basis for egg cell developmental research at the molecular level, and for *in vitro* fertilization. Single cells are essential for manipulation on microchips (Peng *et al*. 2006). The application of molecular biological methods to study the mechanism of egg cell development and fertilization 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*

Table 1. Effect of enzymes on the egg apparatus isolation in the digesting solution. Each result was obtained from 50 ovules and in 1 h.

Pectolyse Y-23 (%)	Cellulase RS (%)	Hemicellulase (%)	Number of released egg apparatuses
0	0	0	1
0.3	1.0	0.8	19
0.6	1.0	0.8	16
0.8	1.0	0.8	15
0.0	1.0	0.8	11
0.3	0	0.8	15
0.3	1.0	0	13

Table 2. Effect of osmolality on egg isolation in dissecting solution. Each result was obtained from 50 ovules and in 1 h.

	6% mannitol (0.401 osmol/kg)	8% mannitol (0.523 osmol/kg)	9% mannitol (0.580 osmol/kg)	10% mannnitol (0.638 osmol/kg)	12% mannitol (0.703 osmol/kg)
Number of isolated eggs	6	14	18	15	9
State of isolated eggs	Inflating	Normal	Normal	Normal	Shrinking

vitro fertilization (Dresselhaus *et al*. 1996). More recently, using a microarray screening technique, Le *et al*. (2005) compared and analyzed gene expression between 25 maize egg cells and 20 central cells. However, these studies were only conducted on maize. The greatest limitation to such molecular characterization in other plants is egg and zygote isolation. In the present study, the number of isolated egg cells of *C. speciosa* was increased to 18 in 1 h, which can reach the cell requirement to permit molecular biological characterization (Okamoto *et al*. 2005) using two rounds of isolation. The protocol provides a simple and effective basis for researching egg development and fertilization in *C. speciosa*.

The two synergid cells characteristic of most angiosperm female gametophytes are specialized to assist in fertilization. Following pollination, the pollen tube enters the ovule through the micropyle, and then proceeds to enter a degenerated synergid. The growth of the pollen tube is arrested in the synergid and the tip breaks to release two sperm cells into the cell. This process initiates fertilization. Many related review papers on the study of synergids have been published, indicating an interest in the role of synergids in plant reproductive biology, but the mechanism of synergids attracting pollen tube growth toward the female gametophyte still remains elusive, which is related to neglected synergid manipulation (Li *et al*. 2009). In the present study, we also isolated a certain amount of synergid cells of *C. speciosa*, which will provide a basis for research as to synergid development, function, and distinguishing features of the two cells.

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