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Isolation of Female Gametes from Ovules of *Torenia baillonii* by Enzymatic Treatments

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Introduction

Recently, techniques have been developed for the isolation and culture of female gametes[1)], and for plant regeneration from *in vitro*-fertilized egg cells of maize[2)] and zygote protoplasts of barley[3)] and maize[4)]. The isolated gametes are expected to be used for various new prospects, such as direct observations of fertilization processes *in vitro*, studies of the mechanism of recognition, adhesion and fusion of gametes, and *in vitro* fertilization studies for breeding. However, it is still difficult to manipulate female gametophytes in most angiosperms, because their development generally takes place deep inside of the sporophytic ovule tissues. So far, enzymatic procedures for isolation of female gametes or embryo sacs have been described in several plant species, such as *Torenia fournieri*[5)], *Lilium longiflorum*[6)], *Zea mays*[7)], *Petunia*[8)] and *Crinum asiaticum*[9)]. Among these species, *T. fournieri* is considered to be an easy species to isolate female gametes because the embryo sac protrudes from the micropyle and can be easily released from intact ovules[5,10)].

The genus *Torenia* belongs to the family Scrophulariaceae and involves many ornamental species. For the breeding of these *Torenia* species, it is now expected to produce interspecific hybrids by conventional as well as biotechnological methods. *In vitro* fertilization is considered to be one of the useful approach to produce the interspecific hybrids in this genus because female gametes of *Torenia fournieri* has already isolated successfully by enzymatic treatments[5)]. In the present study, we developed a method for isolation of female gametes by enzymatic treatments in *T. baillonii* which is considered to be useful traits such as yellow flower color and creeping habit (Fig. 1A).

Materials and Methods

Plant materials and collection of ovules

Flowers of *Torenia baillonii* provided by Takii & Company Ltd. were collected just before anthesis from plants grown in the greenhouse. These flowers were used both for histological observation of the ovules and for isolation of female gametes.

Histological observations of embryo sacs in ovules

After removal of perianths from harvested flowers, ovaries were fixed by FAA solution

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(formalin, acetic acid, 50% ethanol, 5 : 5 : 90, by vol.)[11] for 1-3 days. For the histological studies on ovules and embryo sacs without sectioning, the fixed ovaries were washed by distilled water (D.W.) and stained in modified Mayer's acid haemalaum[12]. After one hour of staining at room temperature, the ovules with placenta were partially destained in D.W. for a period varying from 1 to 3 hours depending on individual staining intensity. Subsequently, ovules with placenta were gradually dehydrated in a series of ethanol solutions, 50% for 2 h, 75% for 2 h and 95% for 24 h successively, and then cleared by successive transfer to 95% ethanol : benzyl benzoate (2 : 1, by vol.), 95% ethanol : benzyl benzoate (1 : 2, by vol.), and lastly to benzyl benzoate : dibutyl phthalate (BBD) (1 : 1, by vol.) at intervals of more than 1 h according to the method of CRANE and CARMAN[13] with several modifications. Treated ovules were picked by forceps from placenta, mounted in BBD on a slide glass with a hollow of 4 mm in depth, covered by a coverslip, and observed by an inverted microscope (IMT-2, Olympus) with Nomarski differential interference equipment.

Isolation of the female gametes by enzymatic treatment of the ovules

After removal of perianths from harvested flowers, ovaries just before anthesis were surface-sterilized with sodium hypochlorite solution (1% active chlorine) for 10 min and rinsed 3 times with sterilized distilled water. The ovaries were longitudinally cut into 2 pieces by a razor blade and ovary walls were peeled off. Two halves of a dissected ovary were put into 2 ml of filter-sterilized (Millipore, 0.45 μm pore size) enzyme solution in a glass Petri dish (35 mm×15 mm). The enzyme solutions used consisted of 2% Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% Macerozyme R-10, 0.05% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 10 mM $CaCl_2 \cdot H_2O$, 5 mM 2-(N-morpholino)-ethanesulfonic acid (MES) and 0.6 M sorbitol. The pH of the enzyme solution was adjusted to 5.8 before filter sterilization. Ovules were then detached from placenta in the enzyme solution by using forceps and a razor blade under a dissecting microscope. They were incubated on a gyratory shaker (30 cycles min^{-1}) at 25 °C. Released female gametes were collected by a micropipette and transferred to CPW solution14) supplemented with 0.6 M sorbitol.

The viability of isolated female gametes was assessed with fluorescein diacetate (FDA) staining[15].

Results and Discussion

Histological observations of embryo sacs in ovules

Microscopic observations showed that the type of ovules was the campylotropous in *T. baillonii* (Fig. 1B) and that the region of egg apparatus in embryo sac protruded from the micropyle. All the cells consisting of the embryo sac were characterized by their location. Approximately half of the elongated central cell located outside the ovule. The antipodal cells were not confirmed in this observation. The structure of embryo sacs in *Torenia baillonii* was similar to that in *T. fournieri* observed by MÓL[5] and KEIJZER *et al.*[16].

Isolation of the female gametes by enzymatic treatment of the ovules

Using enzymatic maceration, we tried to establish a technique for isolation of female gametes from ovules of *T. baillonii*. After 20 min of incubation in the enzyme solution, several cells began to release from the ovules and

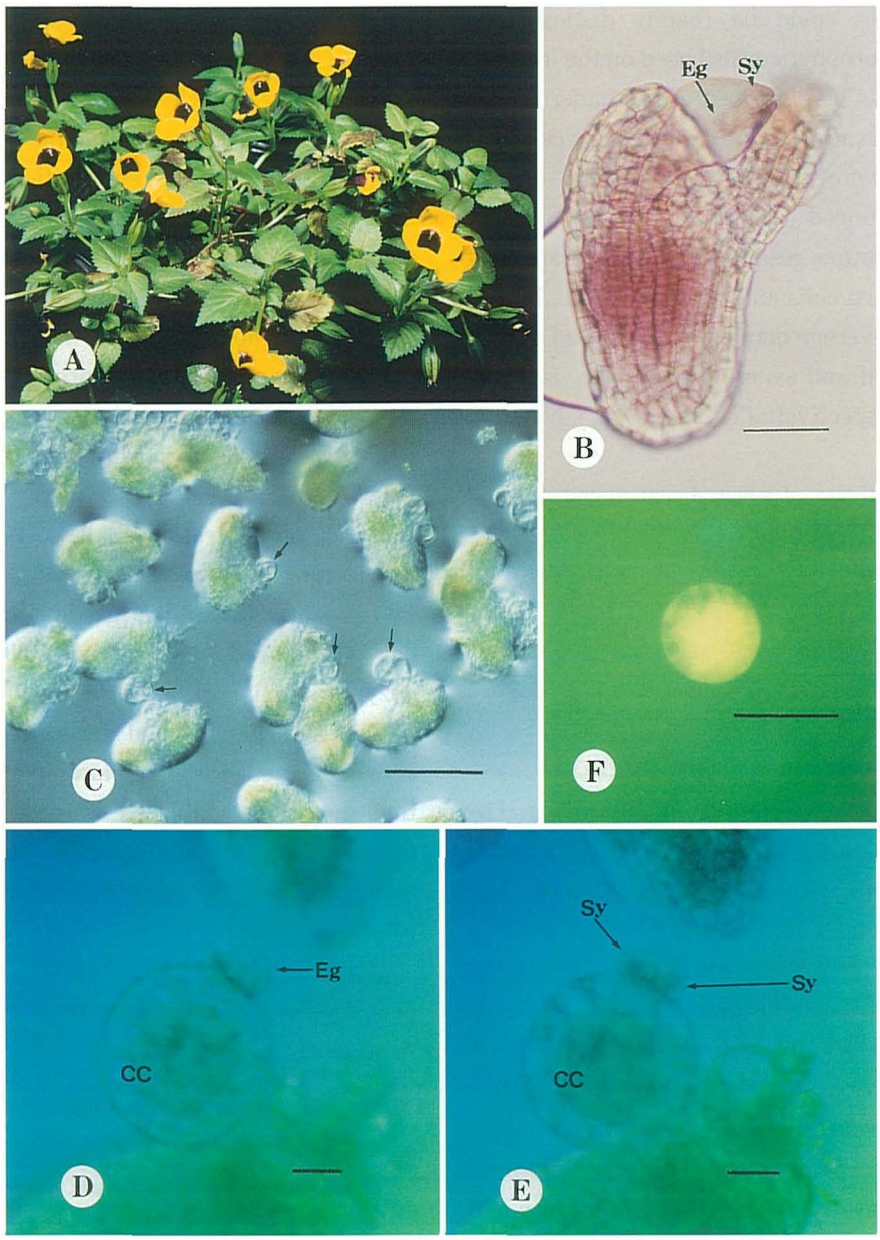

Fig. 1 Morphological characterization of female gametes and isolation of egg cells from ovules of *Torenia baillonii* by enzymatic treatments.
(A) *Torenia baillonii*, the plant material used in this study.
(B) Microscopic observation of embryo sac in ovule under differential interference-contrast optics after staining with acid-haemalaum and clearing with BBD. An egg cell (Eg; arrow) and one of two synergids (Sy ; arrowhead) are in focus. Bar=40 μm.
(C) Ovules treated with enzyme solution for 20 min. Embryo sacs (arrows) were released from ovules. Bar=200 μm.
(D, E) An embryo sac released from ovule after 50 min of enzyme treatment. Egg cell (Eg) and synergids (Sy) focused in D and E, respectively, are releasing from central cell (CC). Bars=15 μm.
(F) FDA-positive egg cell showing the viability. Bar=15 μm.

embryo sacs could be readily distinguished from the sporophytic cells based on the location in the ovules as described above under microscopic observation (Fig. 1C). The egg cells and synergids were still attached to the central cells. Prolonged incubation to 60 min in enzyme solution resulted in the liberation of individual egg cells and synergids (Figs. 1D and 1E). The average diameters of isolated central cell, egg cell and synergid were 40.8, 14.3 and 12.1 μm, respectively (Table 1).

Table 1. Cell size of isolated central cell, egg cell and synergid of T. baillonii

Cell	Diameter (μm)
Central cell	40.8±1.9
Egg cell	14.3±0.7
Synergid	12.1±0.7

The data were obtained by measuring each 30 cells.
Each value represents the mean ± standard deviation.

The egg cells were individually collected by a micropipette for incubation in CPW solution supplemented with 0.6 M sorbitol. The egg cells were viable as assessed by FDA staining even 1 h after the collection (Fig. 1F). However, isolated egg cells gradually lost the viability and the staining entirely disappeared 6 h after the collection. To sustain their viability, it will be needed to modify several factors such as enzyme solutions and culture conditions after isolation. Precise combination of enzymes, pH, osmotic pressure, incubation temperature and incubation time is also required to isolate the embryo sac in a living state.

Thus, we developed a procedure for isolation of female gametes of *T. baillonii* which showed specific morphology of an embryo sac in an ovule. This study also propose the advantage of this plant species as a model system for isolation of female gametes and *in vitro* fertilization.

Summary

Female gametes were successfully isolated from ovules of *Torenia baillonii* by enzymatic treatments. Prior to enzymatic treatments, mature ovules were histologically observed by using clearing method to characterize morphological features of embryo sacs in ovules. The results showed that the region of an egg apparatus in an embryo sac protruded from the micropyle. For the isolation of egg cells, ovules were collected from ovaries of flowers just before anthesis, and treated with enzyme solutions on a gyratory shaker. After 20 min of enzyme treatment, the egg cell accompanied by two synergids was released from the central cell. Prolonged incubation until 60 min in the enzyme solution resulted in the liberation of individual egg cells and synergids. The egg cells collected with a micropipette revealed that they were viable as assessed with fluorecein diacetate (FDA) staining one hour after incubation in CPW solution supplemented with 0.6 M sorbitol. The procedure for isolating egg cells established in this study will offer a new approach to further study on *in vitro* fertilization in this species.

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酵素処理による *Torenia baillonii* の胚珠からの雌性配偶子単離

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摘　要

酵素処理による *Torenia baillonii* の胚珠からの雌性配偶子の単離について検討を行った。はじめに，成熟した胚嚢の形態的特徴を調査するために，透明法による胚珠内部形態の観察を行ったところ，卵装置が珠孔から突出する構造を持つことが明らかになった。雌性配偶子の単離に際し，開花直前の花蕾の子房から胚珠を取り出し，酵素処理を行った。処理20分後には，胚珠から胚嚢が遊離されることが観察された。60分まで酵素処理を延長をすることにより，中央細胞から卵細胞と助細胞の遊離が観察された。遊離した卵細胞をマイクロピペットにより0.6 Mソルビトールを添加したCPW液に回収し，インキュベートを行った。回収1時間後の卵細胞は，FDA染色による調査により活性を保持していることが示された。本研究によって確立された卵細胞の単離技術は，*Torenia baillonii* を用いた試験管内受精の研究に寄与できるものと期待される。