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Original Paper

Direct isolation of female germ units from ovules of *Petunia hybrida* by enzymatic treatment without releasing somatic protoplasts from ovular tissue

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Abstract Establishment of the efficient method for isolating the female germ unit (FGU; egg, synergid and central cell) is useful for the studies on the characterization of each FGU as well as *in vitro* fertilization and gametosomatic hybridization. In this study, an easy one-step enzymatic procedure was successfully developed to isolate FGU from ovules of *Petunia hybrida* without releasing the somatic protoplasts from ovular tissues, which could not be achieved in the previous studies. Each FGU was separately liberated after treating the ovules, which were collected from ovaries of flowers one day after anthesis, with an appropriate enzyme solution comprised of 10 g l^{-1} Cellulase Onozuka R-10, 10 g l^{-1} Macerozyme R-10, 0.6 M mannitol, 5 mM 2-morpholinoethane-sulfonic acid and 5 g l^{-1} potassium dextran sulfate, pH 5.8 with 50-rpm shaking for 2 h at room temperature. Isolated FGUs were distinguished by their specific size and characteristics. Fluorescent staining with 4′, 6-diamidino-2-phenylindole could identify the polar nuclei of central cell and the nuclear polarity of egg apparatus cells. After transfer into washing solution supplemented with 0.6 M mannitol using a micropump-connected microcapillary, about 80% of the isolated FGUs were viable for up to 8 h after the isolation, as determining by fluorescein diacetate staining.

Key words: Egg cell, female germ unit, *Petunia hybrida*, protoplast isolation.

In angiosperm plants, the FGU refers to the minimum complement of cells required to accomplish double fertilization *in vivo* (Dumas et al. 1984; Huang and Russell 1992). Typical FGU consists of an egg, two synergids and a central cell, which fulfills the role of pollen tube attraction, sperm discharge to the receptive cells, transportation of sperm nuclei and double fertilization (Russell 1992; Dumas and Mogensen 1993; Higashiyama et al. 2001). Since the report of embryo sac isolation by Zhou and Yang (1982), the procedures for isolation and culture of embryo sac and female gametophytic cells have been developed over two decades, which has enabled fundamental and applied studies in the area of reproductive biology, such as mechanisms of recognition, adhesion and fusion of gametes, and cellular and molecular changes of gametes in double fertilization process (Dumas and Russell 1992; Faure 2001; Raghavan 2003). Furthermore, isolation of individual FGUs could be utilized to perform some applied studies such as breeding of novel plants through *in vitro* fertilization (Zenkteler 1990; Kranz et al. 1991) and gametosomatic hybridization (Sun et al. 1995).

Until now, female gametophytic cells have successfully been isolated in several monocot species such as *Zea mays* (Kranz et al. 1991; 1998), *Hordeum vulgare* (Holm et al. 1994), *Triticum aestivum* (Kovács et al. 1994), *Oryza sativa* (Khalequzzaman and Haq 2005; Uchiumi et al. 2006) and *Alstroemeria aurea* (Hoshino et al. 2006), as well as in dicot species such as *Vicia faba* (Zhou and Yang 1982), *Helianthus annuus* (Zhou 1987), *Torenia* (Mól 1986; Hoshino and Mii 1999), *Plumbago zeylanica* (Huang and Russell 1989) and *Dianthus* (Hoshino et al. 2000). However, it is still difficult to manipulate FGUs because they develop as parts of embryo sac, which is embedded within the sporophytic tissues of the ovule. The techniques developed so far for isolating female gametes such as embryo sac and free FGUs mostly have several steps comprising the combination of enzymatic maceration and manual procedures (agitation and microdissection) with or without some additional steps such as plasmolysis for facilitating the liberation of embryo sac from ovule or of

Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; FDA, fluorescein diacetate; FGU, female germ unit; MES, 2-morpholinoethane-sulfonic acid; PDS, potassium dextran sulfate.
This article can be found at http://www.jspcmb.jp/

FGU from embryo sac wall (Kranz et al. 1998; Imre and Kristóf 1999). Beside the use of enzymes, Kovács et al. (1994) reported the isolation of viable egg cells of wheat that has been achieved by mechanical disintegration of softened ovule tissues 3 to 7 days after applying 2,4-D to the stigmas.

In *Petunia*, one of the most popular ornamental species in Solanaceae, van Went and Kwee (1990) described the isolation of embryo sacs by enzymatic treatment with subsequent replacing the enzyme solution by a pollen germination medium (Brewbaker and Kwack 1963) with agitation. They also showed that prolonged incubation of ovules or reincubation of isolated embryo sacs in the maceration mixture results in the liberation of gametophytic cells as individual living protoplasts. Since this pioneer study in petunia, there has still been no report on the complete isolation of FGUs in this genus.

In the present study, we aim to develop the enzymatic procedure for the efficient isolation of individual FGUs with the less number of whole embryo sac liberation from the ovule of *P. hybrida* to utilize them for further study on gametosomatic hybridization. Since this one-step enzymatic protocol could isolate only FGUs without degrading somatic ovular tissues, it can overcome the obstacle in discriminating free FGUs from the unnecessary somatic protoplasts.

Materials and methods

Isolation of FGUs

The flowers of *Petunia hybrida* line K2 were collected from plants cultivated in *in vitro* condition 1 day after anthesis. Each whole ovary was put in a 3.5-cm Petri dish and gently squeezed using forceps for releasing ovules in 2 ml of a filter-sterilized enzyme solution supplemented with 0.6 M mannitol, 5 mM 2 (N-morpholino)-ethanesulfonate (MES) and 5 g l^{-1} PDS. For enzyme solution, various enzyme formulae consisting of different combinations and concentrations of Cellulase Onozuka R-10, Macerozyme R-10, Pectolyase Y-23 and Driselase, were tested. The pH of enzyme solutions was adjusted to 5.8 prior to filter sterilization (0.45 μm pore size, Millipore, USA). The dishes with these treatments were incubated on gyratory shaker (50 rpm) for 3 h at room temperature.

Observation and viability test of FGUs

Each isolated component of FGUs (egg, synergid, and central cell) and embryo sac were distinguished from their size and characteristics by observing under an inverted microscope. The nuclei of FGUs were stained with DAPI to identify each component of FGUs from their number and location. The number of isolated each component of FGUs was counted, and the average diameters of FGUs were measured from ten cells of each FGU using an ocular micrometer under an inverted microscope.

Isolated FGUs were collected and transferred into washing solution (0.6 M mannitol, 5 mM MES and 5 g l^{-1} PDS, pH 5.8) by using a micropump-connected microcapillary (Nano Spuit®; IKEDA Scientific Co. Ltd, Japan). The viability of isolated FGUs was assessed with FDA staining (Widholm 1972).

The characteristics of DAPI- and FDA-stained cells were observed under a fluorescence microscope with a UV source at the excitation wavelength of 365 nm and 488 nm, respectively.

Histological observation of ovules

The placentae with ovules were collected from flowers of *in vitro*-cultured plant of *Petunia hybrida* line K2 1 day after anthesis, fixed in a formalin-acetic-alcohol solution, dehydrated through an ethyl alcohol-tertial butyl alcohol series and embedded in paraffin. The sections were cut longitudinally at 16 μm and stained with Delafield's haematoxylin.

Results

Petunia FGUs were isolated from ovules of *in vitro* flower harvested 1 day after anthesis by enzymatic maceration with at least two different enzyme compositions (Table 1). Histological observation of Petunia ovule revealed that embryo sacs are located in the middle of ovules (Figure 1A). After 2 h of treatment with enzyme solution containing 10 g l^{-1} Cellulase Onozuka R-10, 10 g l^{-1} Macerozyme R-10, 5 g l^{-1} PDS, 5 mM MES and 0.6 M mannitol (treatment 7 in Table 1), central cells and egg cells of *P. hybrida* were frequently released directly from 10.3% and 9.4% of total ovules per one ovary, respectively, usually from the micropylar end (Figure 1B) with very less number of embryo sacs (0–1 embryo sac per total ovules of one ovary). Increasing the concentration of Cellulase Onozuka R-10 in enzyme solutions up to 20 g l^{-1}, the amount of isolated egg and central cells decreased, while embryo sacs were frequently liberated. The number of isolated egg and central cells were also decreased when the concentration of Macerozyme R-10 was reduced. High concentration of Cellulase Onozuka R-10 acted inhibitory for the isolation of FGUs (treatment 1–5). Replacement of Macerozyme R-10 by Pectolyase did not favor for isolation of FGUs, which shows that central cells and egg cells could not be obtained in enzyme compositions without Macerozyme R-10 (treatment 8), but a few embryo sacs were isolated. Pectolyase did not favor for isolation of FGUs (treatment 8–10). Moreover, addition of Driselase almost completely inhibited the isolation of embryo sac and FGUs (treatment 10).

The liberated FGUs (egg, synergid, and central cell) and embryo sac were distinguished from each other by their size (Table 2) and characteristics. After enzymatic treatment, the isolated central cells could be obviously distinguished by the large size (*ca.* 68 μm) with the observable cluster of starch granules accumulated around the polar nuclei (Figure 2A). Central cells were also confirmed by DAPI staining of 2 polar nuclei (Figure 2D). Isolated egg protoplasts could be discriminated

Table 1. Percentages of central cell, egg and embryo sacs liberated from ovules of *Petunia hybrida* line K2 after treating with different enzyme compositions for 3 h.

Treatment	Enzyme composition ($g\,l^{-1}$)[a]				Isolated FGUs from ovules per 1 ovary (%)[b]		
	C	M	P	D	Central cell	Egg	Embryo sac
1	30	10	—	—	0.5 ± 0.4 e	0.1 ± 0.3 d	0.7 ± 0.2 ef
2	20	5	—	—	0.5 ± 0.5 e	0.8 ± 0.2 cd	3.3 ± 0.5 ab
3	20	10	—	—	1.3 ± 0.6 de	1.5 ± 0.3 c	4.3 ± 0.7 a
4	15	5	—	—	1.2 ± 0.5 de	1.4 ± 0.7 c	2.9 ± 0.7 bc
5	15	10	—	—	2.4 ± 0.7 cd	2.7 ± 0.5 b	2.1 ± 0.5 cd
6	10	5	—	—	7.3 ± 1.2 b	3.6 ± 0.3 b	1.4 ± 0.3 de
7	10	10	—	—	10.3 ± 0.1 a	9.4 ± 0.9 a	0.2 ± 0.3 f
8	10	—	5	—	0 e	0 d	2.5 ± 0.4 bcd
9	10	10	5	—	3.5 ± 0.5 c	3.5 ± 0.2 b	0.3 ± 0.6 ef
10	10	10	5	5	0 e	0 d	0.2 ± 0.3 f

[a] All enzyme compositions were supplemented with 5 $g\,l^{-1}$ PDS, 5 mM MES and 0.6 M mannitol. C, Cellulase Onozuka R-10; M, Macerozyme R-10; P, Pectolyase Y-23; D, Driselase

[b] Average percentages (±SD.) of central cell, egg and embryo sac isolated from total ovules per one ovary. The different letters within the column show significant difference of each treatment by Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$.

All data obtained from a total of 5 ovaries, the average numbers of ovule per 1 ovary = 181.2 ± 31.0.

Table 2. Cell size of isolated female gametophytic protoplasts of *Petunia hybrida* line K2

Cell	Diameter (μm)[a]
Central cell	68.4 ± 2.2
Egg cell	46.8 ± 3.2
Synergid cell	31.7 ± 2.1
Antipodal cell	37.8 ± 0.8
Embryo sac	94.5 ± 3.3

[a] Average size was measured from 10 cells of each gametophytic cell after enzymatic treatment.

from synergids and the other cells by their cell size (*ca*. 47 μm, Figure 2B). Moreover, DAPI staining showed nuclear polarity of egg cell due to the occupation of its large vacuole (Figure 2E). In contrast, two synergids had no obvious vacuole (*ca*. 32 μm, Figure 2C), and the nucleus located near the center of the isolated protoplast (Figure 2F). They were usually attached to egg cell and released together as a unit of egg apparatus. Egg cell protoplast was detached from egg apparatus prior to the complete separation of synergids into single protoplasts after 2 h of enzymatic treatment. The treatments with different enzyme compositions exhibited the diverse patterns of isolated synergids (Figure 3). In enzyme treatments containing low concentration of Macerozyme R-10 (5 $g\,l^{-1}$, treatment 2, 4 and 6), the liberated synergid pairs could usually not be separated completely from each other, and filiform apparatus remained undigested (Figure 3A) or partially remained at the cell surface (Figure 3B). Isolated synergids from the same ovule were frequently found nearby each other in the enzyme solution (Figure 3C).

Sometimes a cluster of cells usually consisting of three cells was found on the ovule surface apart from micropylar end, which however rarely liberated as protoplasts (Figure 1). These cells were estimated as antipodals (*ca*. 38 μm), since they were obviously larger than somatic protoplasts (*ca*. 16 μm) released from the surface of ovular tissue when unsuitable enzyme solutions were used. Intact embryo sacs (*ca*. 95 μm) could be distinguished from FGUs as the non-spherical cells that were larger than central cell protoplasts, which apparently contained FGUs and antipodal cells inside (Figure 4A). DAPI staining could confirm the 7 nuclei of gametophytic cells within an embryo sac consisting of 3 nuclei of egg apparatus, 3 nuclei of antipodal cells and one secondary nucleus produced by the fusion of two polar nuclei, which was sometimes observed at the stage of flowers used in the present study (Figure 4B).

FGUs were isolated at higher frequencies from *in vitro* flowers harvested 1 day after anthesis than those harvested at anthesis (Figure 5). The number of obtained FGUs slightly decreased 2 days after anthesis, and then drastically decreased at day 3. None of FGUs was released from ovules collected at day 4.

Viability of central cell, egg cell and synergid isolated using the enzyme treatment 7 described in Table 1 was demonstrated by their staining with FDA (Figure 2G, 2H and 2I). More than 80% of isolated FGUs were still viable after the liberation for 3 h in enzyme solution and entirely lost the viability after 4 h. However, they maintained their viability of about 80% for 8 h when they were transferred after 2-h of enzymatic treatment into washing solution consisting of 0.6 M mannitol.

Discussion

The procedures for isolating gametophytic cells in previous reports including that of *Petunia* (van Went and Kwee 1990) were complicated, time-consuming and necessary to isolate the embryo sac from the ovule prior to obtaining FGUs after digesting or removing somatic tissues surrounding embryo sac. In this study, we successfully developed an appropriate enzyme formula

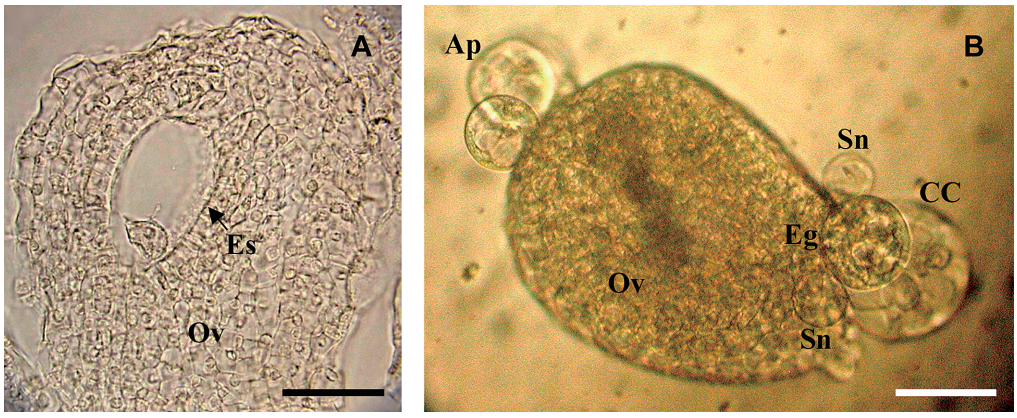

Figure 1. Release of female germ units from ovule of *Petunia hybrida* line K2 by enzymatic treatment. (A) Longitudinal section of an ovule showing the embryo sac in the center. (B) An ovule with gametophytic cells liberated after 90 min of enzymatic treatment with the composition of treatment 7 (Table 1). Ap, 3 antipodals; CC, central cell; Eg, egg cell; Es, embryo sac; Ov, ovule; Sn, synergids. Bar=50 μm.

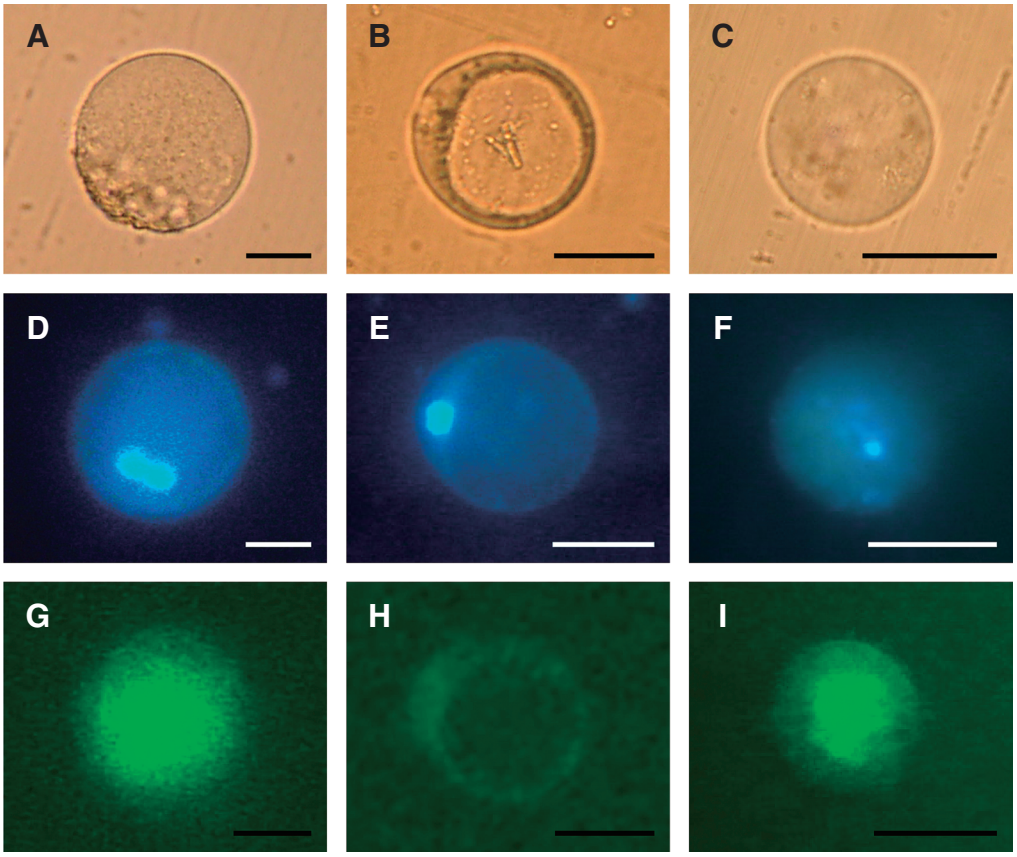

Figure 2. FGUs (central cell, egg and synergid protoplast) isolated from ovules of *Petunia hybrida* line K2 by enzymatic treatment. Bar=25 μm. (A) Central cell protoplast isolated from ovule after 2 h of enzymatic treatment. (B) Egg protoplast isolated from ovule after 2 h of enzymatic treatment. (C) Synergid protoplast isolated from ovule after 2 h of enzymatic treatment. (D) DAPI staining illustrated 2 polar nuclei of central cell protoplast. (E) DAPI staining of egg protoplast showing polarized nucleus. (F) DAPI staining of synergid protoplast. (G) FDA-positive central cell protoplast showing viability after 3 h of enzymatic treatment. (H) FDA-positive egg protoplast showing viability after 3 h of enzymatic treatment. (I) FDA-positive synergid protoplast showing viability after 3 h of enzymatic treatment.

for one-step isolation of individual FGUs without digesting the surrounding ovule tissues in *P. hybrida* within 2 h. This enzymatic procedure could avoid the difficulty to identify and collect the isolated FGUs among the numerous protoplasts released from somatic ovular tissues. Ten treatment protocols of enzymatic maceration were evaluated, with at least two different variations of enzymes in each formula that mainly composed of Cellulase and Macerozyme. The most favorable result was obtained using a solution containing

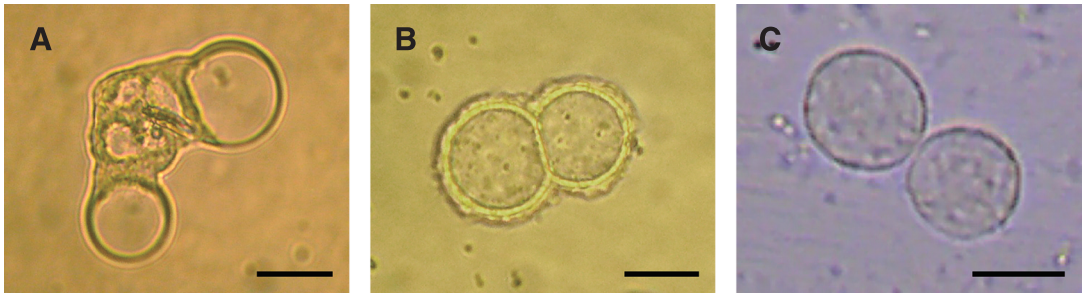

Figure 3. Synergids isolated from ovules of *Petunia hybrida* line K2 after incubation for 2 h in different treatment of enzyme formula. Bar=25 μm. (A) Two synergids attached with filiform apparatus occasionally released in enzyme treatment 6. (B) A pair of synergids adhered together with remaining undigested filiform tissue in enzyme treatment 4. (C) Isolated synergids in enzyme treatment 7.

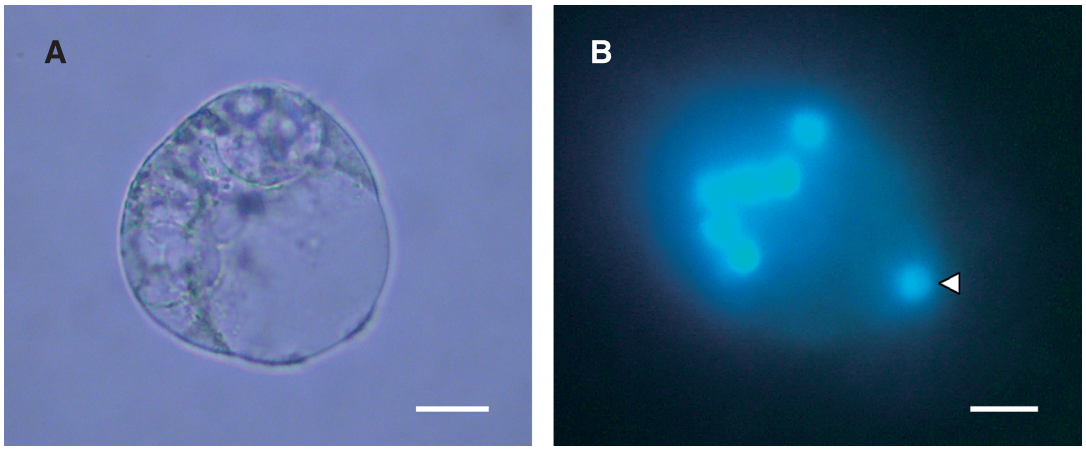

Figure 4. Embryo sac isolated from an ovule of *Petunia hybrida* line K2 after 2 h of enzymatic treatment. Bar=25 μm. (A) Embryo sac containing FGUs (central cell, egg and synergids) and antipodal cells. (B) The same embryo sac stained with DAPI embryo sac showing nuclei of 1 central cell (arrow; secondary nucleus derived from fusion of polar nuclei), and closely associated 6 nuclei corresponding to 1 egg, 2 synergids and 3 antipodal cells, which are difficult to identify each other.

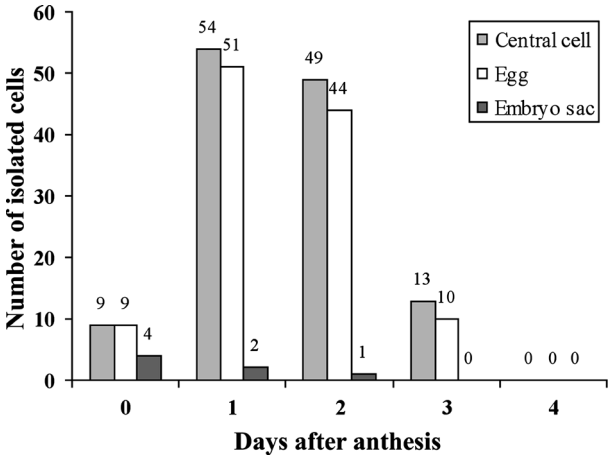

Figure 5. Yield of female gametophytic protoplasts of *Petunia hybrida* line K2 isolated by enzymatic treatment 7 using 3 ovaries (181.2±31.01 ovules/ovary) collected on different days after anthesis.

10 g l^{-1} Cellulase Onozuka R-10, 10 g l^{-1} Macerozyme R-10, 0.6 M mannitol, 5 mM MES and 5 g l^{-1} PDS (treatment 7). This enzyme formula might moderately digest and puncture the ovular tissue of embryo sac in the appropriate degree, by which FGUs could directly penetrate out from the ovules within 2 h of treatment, whereas the nucellus residue of ovule still remained inside. The success of FGU isolation of treatment 7 might be due to the optimum proportion of Cellulase-Macerozyme combination for *P. hybrida* ovule and the presence of PDS in the enzymatic mixture.

Macerozyme is a pectin-degrading enzyme normally used for isolation of living single cells from leaves and cultured plant tissues (Toyama 1965; Takebe 1968). In previous reports on female gamete isolation, the application of pectinase such as Macerozyme was quite rare although cellulase was regularly contained in enzyme solutions. In *Torenia fournieri* (Mól 1986) and *Nicotiana tabacum* (Fu et al. 1997), success in isolating FGUs including central cell was reported by using enzyme solution containing Macerozyme R-10. In the present study, FGUs were usually emerged from micropylar end of the ovules in treatment 7, in which the cooperative activity of Cellulase and Macerozyme was assumed to degrade the pectic middle lamella between embryo sac and nucellus cells at the thinnest layer around filiform apparatus, which occupies the utmost region of micropyle. After dissociating the cell junctions

and extending intercellular spaces, enzyme solution could well penetrate and digest the cell wall of nucellus and the filiform apparatus, which shows the loosely organized cellulosic network, embedded in a thick pectin matrix protruding into the cytoplasm of two synergids (reviewed in Yang 2006).

Replacement of Macerozyme by Pectolyase resulted in no release of FGU but caused digestion of surrounding ovular tissues and release of a few embryo sacs. This suggests that the nucellus layer at micropyle and filiform apparatus has different property in pectic substances of the cell wall from those of integument tissues. In the previous study on P. hybrida (van Went and Kwee 1990), single application of Driselase at $30\,g\,l^{-1}$ was successfully used for isolating embryo sacs. However, it gave inhibitory effect on isolation of both FGUs and embryo sacs even at lower concentration ($5\,g\,l^{-1}$) when used with Cellulase, Macerozyme and Pectolyase, although the reason is not clear.

While digestive enzymes were essential to break down the major cross-linking elements of cell walls and filiform apparatus, PDS played an important role for the isolation of FGUs in the present study. PDS has widely been used for pollen protoplast isolation (Tanaka et al. 1987; Zhang et al. 2004), protoplast maintenance (White and Overall 1989) and embryo sac isolation (Zhou and Yang 1985) mainly for increasing the yield of intact protoplasts (Wakasa 1973), reducing the damage of tissue dissociation and the toxicity of enzyme to the cells (Takebe and Nagata 1984), and improving viability of isolated protoplasts by protecting oxidative stress (Aoyagi et al. 2002). In the present study, we found that the absence of PDS in enzyme solution, egg and synergid cells could not properly separate from each other and fused together incidentally, and the isolated FGUs usually adhered to the Petri dish bottom, causing them difficult to manipulate. Accordingly, in this study, PDS favored for the isolation of intact FGUs through its anti-adhesion property.

Previous researches have reported that enzymatic treatment adversely affected survival (Wagner et al. 1989; Huang and Russell 1992; Hoshino et al. 2000) and functions (Holm et al. 1994; Leduc et al. 1995) of isolated female gametophytic cells probably because of the high enzyme concentrations applied in these studies. In the present study, high concentration ($15–30\,g\,l^{-1}$) of cellulase also gave more damage to isolated FGUs, although they were released more and faster than $10\,g\,l^{-1}$ (treatment 7). In contrast, FGUs isolated from the treatment 7 could remain alive after the liberation for 4 h in enzyme solution with over 80% viability, and their viability could be longer maintained for 8 h if they were transferred from 2-h enzymatic treatment into the washing solution supplemented with 0.6 M mannitol, without nutrient. Therefore, combination of the short isolation period, the moderate enzyme concentration and appropriate condition seems to produce the high quality and quantity of viable isolated FGUs.

In petunia, ovules could be easily collected by squeezing the surface of ovary using forceps, without micro-dissecting the ovary by razor blade. The squeezing treatment might sometimes have given the wounding of the opposite site of ovule surface to FGUs releasing site at micropylar end, which might be the reason for the occasional liberation of antipodals.

The yield of isolated FGUs also depended on the timing of ovule collection around anthesis. Embryo sac generally enters its maturity after anthesis at nearly the same time that the pollen tube could reach the embryo sac for effecting fertilization, which relates to the long duration of pollen tube growth through the length of the style. Tian and Russel (1997) described that tobacco female gametophytes are usually immature at the day of anthesis and that some of embryo sacs have far fewer cells than the full complement of eight nuclei. Therefore, in *N. tabacum* with approximately 4-cm length of style, female gametophytic cells are available to isolate at 1 day after anthesis and has the optimum conditions for fertilization on the next day. Consistently, in *P. hybrida*, we found that the *in vitro* flowers at 1–2 day after anthesis were suitable to obtain a fair amount of the individual FGUs comparing with at anthesis.

Techniques for direct isolation of FGUs of *P. hybrida* with very low release of embryo sacs and their characterizations demonstrated in this study are advantageous to study further on fundamental and applied plant researches of individual female gametophytic cells that cannot be done by using isolated embryo sac, such as culture and regeneration of FGU protoplasts, transformation and direct gene transfer of FGUs, *in vitro* fertilization, and gametosomatic hybridization. Moreover, the isolation efficiency of this enzymatic protocol could also be useful for the experiments that need high quantity of samples such as biochemical and molecular analysis of FGUs.

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