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Original Paper

Gametosomatic hybridization between egg cell protoplast and mesophyll protoplast of *Petunia hybrida*

Ratchada Sangthong[1], Dong Poh Chin[1], Kanyaratt Supaibulwatana[2], Masahiro Mii[1,*]

[1] Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan; [2] Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand
* E-mail: miim@faculty.chiba-u.jp Tel & Fax: +81-47-308-8852

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Abstract Gametosomatic hybridization has so far been achieved by the fusion between male-gametic (microspore tetrad or young-stage pollen) protoplasts and somatic protoplasts but no successful results have been reported on the use of female gamete so far. In this paper, we demonstrate for the first time the successful gametosomatic hybridization by using female gametophyte (egg cell) as the gametic-haploid partner instead of male gametophyte based on the method established for isolation of female germ units from ovules in *Petunia* in our previous report. Using *P. hybrida* strains, each single protoplast from egg cell and mesophyll cell were manually collected by a micropump-connected microcapillary and were then fused together as an individual pair by electrofusion. Each heterokaryon thus produced was transferred into Millicell culture plate placed in a Petri dish containing nurse cells, where cell division and microcolony formation took place. Among the fusion combinations tested, only fusants between mesophyll protoplast and egg cell protoplast from the same *Petunia* strain could regenerate and develop into the complete plants, whereas inter-varietal combinations failed to grow after developing into microcolonies or to regenerate plants. The flowers of a triploid hybrid line ($2n=3x=21$), confirmed by flow cytometric analysis and chromosome observation, were smaller and shorter than those of the parent with original color, and showed male sterility. These results indicate the intactness of isolated female gametophyte protoplasts, which might enable not only to produce triploid plants with various genomic combinations through gametosomatic hybridization but also to conduct fundamental studies on *in vitro* fertilization with isolated sperm cell protoplasts.

Key words: Egg cell protoplast, gametosomatic hybridization, male sterility, protoplast fusion, triploid hybrid.

Successful isolation of gametophytic cells (Bhojwani and Cocking 1972; Tanaka et al. 1987; Huang and Russell 1989) enabled us to utilize gametophytic-cell protoplasts as one of fusion partners in asymmetric hybridization experiments to produce the novel lines of agricultural importance. In the first relevant report (Pirrie and Power 1986), gametosomatic hybrid plants were regenerated following the fusion between mesophyll protoplasts of a nitrate reductase-deficient mutant of *Nicotiana tabacum* ($2n=4x=48$) with *N. glutinosa* tetrad protoplasts ($n=x=12$). The resulting plants were confirmed as pentaploid gametosomatic hybrids, which had functionally triploid chromosome number ($2n=5x=60$). Therefore, tetrads are considered as a generally available source of haploid protoplasts for gametosomatic fusion studies. Since then triploid gametosomatic hybrids have been produced by the fusion between tetrad protoplasts and somatic protoplasts in *Petunia hybrida* in both intra- and interspecific levels (Lee and Power 1988a; 1988b) and in interspecific combinations of *Nicotiana* (Pental et al. 1988; Giddings and Rees 1992). Intergeneric gametosomatic hybridization has also been reported in Solanaceae, between *N. tabacum* mesophyll protoplasts and *P. hybrida* tetrad protoplasts (Choi et al. 1992).

Isolation of male gamete protoplasts has also been achieved from young pollen at mid-late unicellular to early-middle bicellular stages. Li et al. (1994) reported the first successful result in gametosomatic hybridization by using pollen protoplasts of *Brassica chinensis* rather than tetrad protoplasts as the gametophytic partner for fusing with hypocotyl protoplasts of *B. napus*. By this success, targets for gametosomatic hybridization have been extended to the other families than Solanaceae. However, when they proceeded to a related study using hypocotyl protoplasts of *B. juncea* instead (Li et al. 1996), hybrid calli failed to regenerate shoots and only differentiated roots. In *Nicotiana*, interspecific gametosomatic hybrids could also be achieved by using pollen protoplasts for fusing with somatic protoplasts (Desprez et al. 1995; Lu et al. 1996).

Despite of the success in isolation of female germ units in *P. hybrida* (van Went and Kwee 1990; Sangthong

Abbreviations: BAP, 6-benzylaminopurine; DAPI, 4,6-diamidino-2-phenylindole; FDA, fluorescein diacetate; FGUs, female germ units; NAA, α-naphthaleneacetic acid; PDS, potassium dextran sulfate
This article can be found at http://www.jspcmb.jp/

et al. 2009) and gametosomatic hybridization using male gametophytic protoplasts as gametic donor (Lee and Power 1988a; 1988b; Choi et al. 1992), female gamete has not so far been used for gametosomatic hybridization. The purpose of this study is to achieve the triploid gametosomatic hybrids from the fusion between egg cell protoplast (haploid female gamete) and somatic-mesophyll protoplast of *P. hybrida* strains by using electrofusion technique aiming to demonstrate the usefulness of egg cell in gametosomatic hybridization to produce novel plant genotypic material.

Materials and methods

Plant materials

Two lines of *Petunia hybrida*, K2 (light-pink color) and C2 (pink-purple color), which were pure lines established at our laboratory, and one cultivar 'Dainty Lady' (DL; vivid-pink color), which was kindly provided by Sakata Seed Co., were used as plant materials. One plant was selected from each line and cultivar and they have been maintained in the greenhouse by repeated cuttings.

Isolation and culture of mesophyll protoplasts

Petunia plantlets were cultured *in vitro* on 8 g l^{-1} agar-solidified MS (Murashige and Skoog 1962) basal medium containing 30 g l^{-1} sucrose at pH 5.8, and maintained by subculturing at 4 week-intervals. Fully expanded leaves of *in vitro*-grown petunia plantlets (0.5 g) were cut into pieces and soaked in 5 ml of washing solution (WS) containing 0.6 M mannitol and 5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 5.8) for 1 h in the dark at room temperature for inducing plasmolysis. Then, WS was replaced with 5 ml of a filter-sterilized enzyme solution consisting of 15 g l^{-1} Cellulase Onozuka R10, 5 g l^{-1} Macerozyme R10 (both Yakult Pharmaceutical Ind. Co. Ltd., Japan), 0.6 M mannitol and 5 mM MES, pH 5.8. After incubation in darkness at room temperature with gentle shaking (40 rpm) for 5 h, the mixture was filtered through a nylon sieve (60 μm mesh). Isolated protoplasts were washed with WS, and purified by floatation on 0.6 M sucrose solution with centrifugation (120×*g* for 3 min). The protoplasts were then washed twice by centrifugation in WS. In average, 8×10^6 protoplasts were obtained from 1 g leaves.

Isolation of egg cell protoplasts

The procedure for isolation of egg cell was the same as that established in our previous study (Sangthong et al. 2009). Briefly, ovaries were collected from *in vitro* flowers 1 day after anthesis. Each whole ovary was put in a 3.5-cm Petri dish and gently squeezed for releasing ovules in 2 ml of a filter-sterilized enzyme solution containing 10 g l^{-1} Cellulase Onozuka R10, 10 g l^{-1} Macerozyme R10, 0.6 M mannitol, 5 mM MES and 5 g l^{-1} potassium dextran sulfate (PDS), pH 5.8. After incubation in the dark at room temperature with 50 rpm shaking for 2 h, the liberated egg cell protoplasts were selected from the mixture of female gamete cells by a microcapillary connected to a micropump (Nano Spuit®; IKEDA Scientific Co. Ltd, Japan) and placed into WS prior to fusion pretreatment.

Isolated egg cell protoplasts were identified by nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) and assessed for the viability using fluorescein diacetate (FDA) staining (Widholm 1972). The stained cells were observed with a fluorescence microscope (Olympus BX60) with a UV filter.

Fusion, culture and regeneration

After obtaining mesophyll protoplasts and egg cell protoplasts, Nano Spuit® was used for collecting individual protoplast into 200 μl of fusion solution (1 mM $CaCl_2\cdot 2H_2O$, 5 mM MES and 0.6 M mannitol, pH 5.8) in electrofusion chamber with 4 mm electrode gap (ETC-04, BTX, USA). Electrofusion was carried out using an Electro Cell Manipulator 200 (BTX, USA). One-by-one fusion between each one mesophyll protoplast and egg cell protoplast was mediated by the application of an AC field of 2 MHz at 100 V cm^{-1} for 10 s followed by a DC pulse developing 1250 V cm^{-1} for 40 μs. After fusion was completed, each fusion product was collected and placed (1–7 fusants) into a Millicell® tissue culture plate (Millicell-CM, 12 mm; Millipore, USA), which was kept in a 3.5 cm plastic Petri dish of nurse cell protoplasts of *P. hybrida* line C2 suspended at a density of 1×10^5 protoplast ml-1 in protoplast liquid culture medium; half strength-macronutrient MS liquid medium supplemented with 1 mg l^{-1} α-naphthaleneacetic acid (NAA), 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 0.5 M mannitol and 0.1 M sucrose, pH 5.8.

When fused cells grew into microcolonies of ca. 1 mm in diameter, they were transferred onto callus proliferation medium; 2 g l^{-1} gellan gum-solidified MS medium supplemented with 2 mg l^{-1} NAA, 0.5 mg l^{-1} BAP and 30 g l^{-1} sucrose, pH 5.8. After two months of culture on callus proliferation medium, calli thus obtained were transferred for inducing shoots on 1/2 MS medium containing 0.01 mg l^{-1} NAA, 0.5 mg l^{-1} BAP, 15 g l^{-1} sucrose and 0.2 g l^{-1} gellan gum, pH 5.8. Regenerated shoots were excised from callus and propagated by subculture on hormone-free MS medium containing 30 g l^{-1} sucrose and solidified with 0.8 g l^{-1} agar, pH 5.8.

Flow cytometric analysis

Flow cytometry was applied to quantify DNA content of the fusion products according to the method of Mishiba and Mii (2000) using a Partec PA cytometer equipped with a mercury lamp (Partec, Germany). Ploidy level was determined by comparing the position of dominant peaks corresponding to nuclei at G0-G1 phase of the cell cycle, between fusion product and normal diploid plant. To release nuclei, approximately 0.1 g fresh weight of the sample tissues was collected and chopped with a razor blade in 0.3 ml of solution A of plant high-resolution DNA kit type P (Partec, Germany) in a plastic Petri dish (Galbraith et al. 1983). After incubating the crude nuclei suspension for 5 min at room temperature, 1.5 ml of staining solution composed of 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 10 g l^{-1} PVP, 1 ml l^{-1} Triton X-100 and 2 mg l^{-1} 4′,6′-diamidino-2-phenylindole (DAPI), pH 7.5, was added to the crude suspension and filtered through a 30-mm nylon mesh. After 5 min of staining, the suspension of nuclei was subjected to FCM analysis for determining the relative nuclear DNA content on a semi-logarithmic scale histogram.

Chromosome observation

Chromosome number was determined by treating root tips of *in vitro* plantlets with 0.5 mg l^{-1} amiprophos methyl for 1.5 h at room temperature, with subsequent fixation in a mixed solution of ethanol: glacial acetic acid (3 : 1) for at least 1 h. After rinsing with distilled water, the fixed root tips were then macerated in enzyme solution composed of 20 g l^{-1} Cellulase Onozuka RS, 15 g l^{-1} Macerozyme R200 (both Yakult, Japan), 3 g l^{-1} Pectolyase Y-23 (Seishin, Japan) and 1 mM EDTA, pH 4.2 at 37°C (Yamamoto and Tominaga 2004) for 30 min. The root tips were immersed in 10 g l^{-1} acetocarmine solution for at least 15 min for chromosome staining. Each root tip was squashed under few drops of 45% acetic acid on the slide glasses. Chromosomes were observed and taken photographs under microscope.

Pollen fertility

Pollen fertility of a fusion-derived plant was examined comparing with normal pollen grains of parental plant. Pollen viability was assessed by staining nuclei with DAPI, and observed fluorescing nuclei contained in pollen grains under fluorescence microscope with an ultraviolet source. Pollen of parental line and hybrid were cultured in pollen germination medium (PGM) containing 0.1 g l^{-1} H_2BO_3, 0.3 g l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 0.3 M sucrose (Scott 1995) adjusted to pH 5.8, and the pollen germination exhibiting normal pollen tube growth was examined.

Results

Isolation of egg cell protoplasts

Egg cells were successfully isolated from ovules by enzymatic maceration at a 9.4% yield of the total ovules. The liberated FGUs (egg, central cell, synergids, antipodal and embryo sac) were distinguished from each other by their size and characteristics (Figure 1A). After enzymatic treatment, egg cells could be obviously recognized by their large-vacuole and cell size (ca. 47 μm, Figure 1B). DAPI-staining showed nuclear polarity of egg cell due to its large vacuole (Figure 1C) as shown in our previous study (Sangthong et al. 2009). Viability of isolated egg cell protoplasts was demonstrated by their staining with FDA (Figure 1D). Eighty percent of isolated egg cells were still viable after the liberation for 3 h in enzyme solution but they entirely lost the viability after 4 h. In contrast, they maintained their viability for 8 h in washing solution supplemented with 0.6 M mannitol after transfer from 2-h enzymatic treatment.

Gametosomatic hybridization and plant regeneration

Both egg cell protoplast and mesophyll protoplast were singly collected and transferred into an electrofusion chamber using nano-tube. Each pair of them was electrofused one by one (Figure 2A). Fusion products (Figure 2B) cultured in Millicell® with surrounding nurse cells frequently showed budding formation after 4-5 days of culture. The initiation of division in fusant (Figure 2C) rather delayed (day 10th–16th) when compared with normal mesophyll protoplasts (day 3rd–4th). The sustained divisions gave rise to microcolonies after 30–45 days (Figure 2D) and formed visible colonies on the membrane filter of Millicell® (Figure 2E) after 60 days of culture. Plating efficiency of the fusants estimated after 1.5 months of culture was varied according to the fusion combinations and the highest plating efficiency (25%) was obtained in the fusants between mesophyll protoplast and egg cell protoplast of the same K2 line (K2E hybrids) (Table 1). Although the calli of this fusion combination and those produced by the fusion between DL mesophyll protoplast and K2 egg cell protoplast (DKE hybrids) continued to proliferate as callus clumps, micro-colonies of the other hybrid combinations between different lines (CKE and KCE hybrids) could not grow well and gradually died after colony formation.

After 2 months of culture, growing calli of fusants (~1 mm) were transferred onto callus proliferation medium, on which they became bigger clumps during 1–2 months of culture. They were then transferred onto shoot regeneration medium, on which shoot regeneration initiated to occur after 3 months in 4 out of 12 K2E hybrid calli (Figure 2F). The hybrid plantlets showed hyperhydricity at the early stage of regeneration (Figure 2G), but gradually became normal during the prolonged *in vitro* culture period for at least 4 months. Among the 4 regenerable lines, only one triploid hybrid line (K2E-1) produced flowers after the recovering from abnormal characters (Figure 2H). In contrast, no shoot was obtained from DKE hybrid calli (Table 1).

Cytological analysis

Flow cytometric analysis of relative nuclear DNA content of *P. hybrida* K2 and the gametosomatic hybrids was conducted after staining nuclei with DAPI by adjusting the peak of 2C value K2 to the channel 50 (Figure 3A). Among the 12 K2E hybrid calli obtained, a hybrid plant (K2E-1) regenerated from one callus line showed the dominant peak at the channel 75, which corresponded to the expected triploid ($2n=3x=21$) value resulting from the sum of somatic cell and egg cell DNA contents (Figure 3C). A hybrid plant (K2E-8) from another callus line revealed 2C value at channel 65 (Figure 3E). This was considered to be aneuploid with estimated chromosome number of $2n=18$. Regenerated plantlets from another two regenerable callus lines showed the peak at channel 50 same as diploid control plant. Among the 8 unregenerated calli, most of them (7 callus lines) showed the dominant peak as diploids. However, one hybrid callus line (K2E-C5) showed 2C value around 65–70 which was considered to be aneuploid with estimated chromosome number $2n=18$

Table 1. Cell division, plating efficiency and plant regeneration in the gametosomatic hybridization between egg cells and mesophyll protoplasts of *Petunia hybrida*

Hybrid Codes	Fusion combinations: Mesophyll protoplasts	Fusion combinations: Egg cells	No. of fusant	Plating efficiency (%)[a]	No. of calli (%)[b]	No. of fusants with shoot regeneration (%)
K2E	K2	K2	51	25.4±8.0	12 (23.5)	4 (7.8)
DKE	DL	K2	28	14.1±5.1	1 (3.6)	0
CKE	C2	K2	35	15.9±11.0	0	0
KCE	K2	C2	30	10.3±9.2	0	0

[a] Percentage (±SD.) of colony-forming fusants in a millicell (12 mm in diameter) estimated after 1.5 months of culture.
[b] DKE hybrid lines could not regenerate, and have been maintained as callus clumps. CKE and KCE hybrids gradually died after microcolony formation.
Number in parenthesis indicated the percentage of fusion products that were able to perform each character.

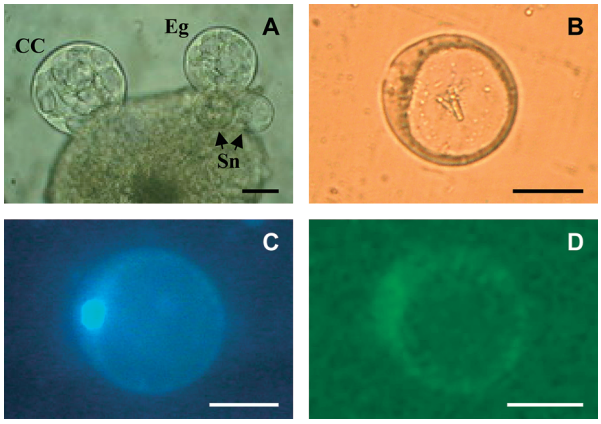

Figure 1. Morphological characteristic of egg cells isolated from an ovule of *P. hybrida* line K2 by enzymatic treatment. Bar=25 μm. (A) Female germ cells released from ovule after 90 min of enzymatic treatment. CC, central cell; Eg, egg cell; Sn, synergids (arrows). (B) Egg cell isolated from ovule after 2 h of enzymatic treatment. (C) DAPI staining of egg cell showing polarized nucleus. (D) FDA-positive egg cell showing viability liberated after 3 h of enzymatic treatment.

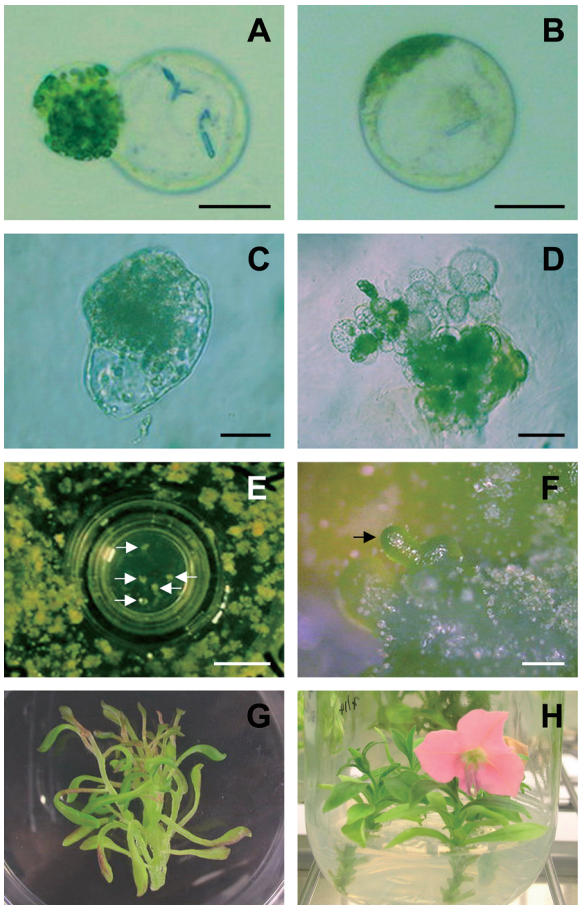

Figure 2. Gametosomatic hybridization between mesophyll protoplast and egg cell protoplast of *P. hybrida* line K2. (A) Adhesion of mesophyll protoplast (left) and egg cell (right). Bar=25 μm. (B) Gametosomatic fusant produced after electrofusion treatment. Bar=25 μm. (C) Cell division of fusant after 10 days of culture. Bar=25 μm. (D) Microcolony formation after 1.5 months of culture. Bar=100 μm. (E) Visible colonies (arrows) of fusants grown in millicell surrounding with nurse cells derived from mesophyll protoplasts of *P. hybrida* (1×10^5 cells/ml). Bar=5 mm. (F) Shoot regeneration (arrow) after 6 months of culture. Bar=0.5 mm. (G) Hyperhydric shoot of regenerated plantlet of triploid hybrid line K2E-1 ($2n=3x=21$). Bar=1 cm. (H) Regenerated plant with flower of gametosomatic hybrid line. Bar=1 cm.

(data not shown).

Chromosome counting made on root tip cells revealed that the parental line K2 had diploid chromosome number of $2n=2x=14$ (Figure 3B). Whereas, K2E-1 hybrid derived from the fusion between egg cell and mesophyll cell of K2 had triploid chromosome number of $2n=3x=21$ (Figure 3D), and K2E-8 hybrid was aneuploid with chromosome number $2n=18$ (Figure 3F) as expected.

Characterization of hybrid plants

After regeneration of K2E-1 triploid hybrid, some morphological characteristics of K2E-1 were compared with those of the parental plant K2. Although K2E-1 produced flowers with the same light-pink color as the parent, the size of the flowers of K2E-1 was smaller and shorter (1-cm length, 2.5-cm breadth) than the original plant (2 to 2.5-cm length, 3.5 to 4-cm breadth), (Figure 4A, B).

Diploid parental plant K2 produced normal fertile pollen, which showed germination on culture medium

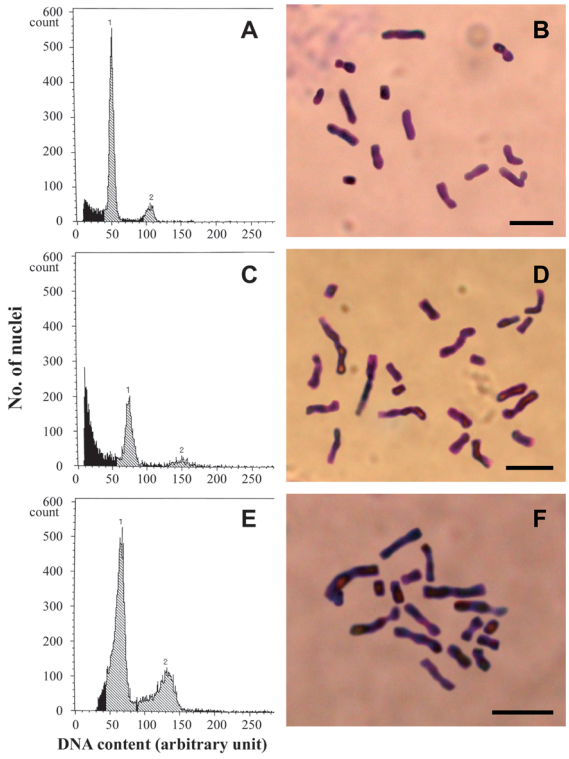

Figure 3. Flow cytometric histograms and chromosomes of *P. hybrida* line K2 and gameto-somatic hybrids obtained from fusion between egg cell and mesophyll protoplast. Bar=5 μm. (A, B) Control line ($2n=2x=14$). (C, D) Triploid hybrid line K2E-1 ($2n=3x=21$). (E, F) Aneuploid hybrid line K2E-8 ($2n=18$).

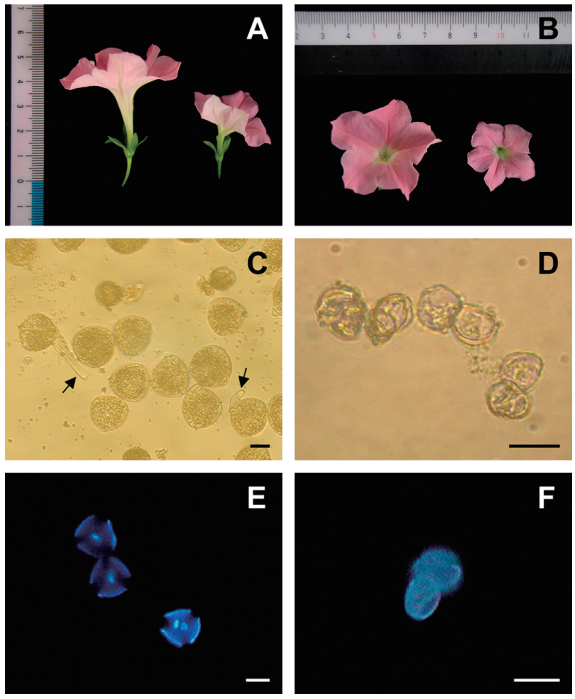

Figure 4. Characteristics of flowers and pollen of *P. hybrida* line K2 and gameto-somatic hybrids obtained from fusion between mesophyll protoplast and egg cell. Bar=50 μm. (A, B) Flowers of control line ($2n=2x=14$) and triploid hybrid line K2E-1 ($2n=3x=21$), left to right. (C) Pollen of control line with tube germination (arrows) after 30 min of culture in medium containing 0.1 g l^{-1} H_2BO_3, 0.3 g l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 0.3 M sucrose. (D) Abortive pollen of triploid hybrid line K2E-1. (E) Normal pollen grains containing DAPI-stained nuclei. (F) Pollen grains of triploid hybrid line K2E-1 showing no stained nuclei with DAPI.

and positive nuclei staining with DAPI (Figure 4C, E), whereas pollen grains of triploid hybrid line K2E-1 were shriveled, empty, and smaller in size with no stained nuclei with DAPI (Figure 4D, F), indicating their sterility.

Discussion

In this paper we for the first time report gametosomatic-hybrid production between female gamete (egg cell) and mesophyll protoplast of *Petunia hybrida* by using electrofusion. Attempts to produce asymmetric hybrids between the genome of gamete and that of somatic cell, was first reported by Pirrie and Power (1986), which have been followed by several groups describing the studies of gametosomatic hybridization in Solanaceae (*Nicotiana* and *Petunia*) and Brassicaceae (*Brassica*). However, all of the researches that have done these studies used male gametophytic protoplasts (tetrad and young stage pollen protoplasts) as gametic donor. Among these, gametosomatic hybridization in *Petunia* has been reported in both intra- and interspecific levels (Lee and Power 1988a, 1988b) using polyethylene glycol (PEG) and high pH-induced fusion. The triploid gametosomatic hybrid plants possessing intermediate vegetative and floral morphologies could be obtained from both intraspecific-fusion between tetrad protoplasts of a normal purple flowered variety of *P. hybrida* and cell suspension protoplasts of a nuclear albino mutant of the variety Blue Lace, and interspecific fusion between tetrad protoplasts of the same normal *P. hybrida* and cell suspension protoplasts of a nuclear albino mutant of *P. parviflora*. Hybrid cells in both systems were identified as green colonies against an albino background as a result of complementation to chlorophyll proficiency. Moreover, in intergeneric level, Choi et al. (1992) reported gametosomatic hybridization through the fusion between *N. tabacum* mesophyll protoplasts and *P. hybrida* tetrad protoplasts. These results clearly indicate that male gametophytic cell of *Petunia* has the ability to produce gametosomatic hybrids, which sometimes showed totipotency. However, the ability of female gametophytic cells to interact with somatic cells in the gametosomatic hybrid condition has not been reported yet. In the present study, therefore, we attempted to demonstrate hybrid feature between female gametophytic and somatic protoplasts of *P. hybrida* by fusing egg cell protoplasts with mesophyll protoplasts.

In most angiosperms, it is still difficult to manipulate female gametophytes because they are generally

embedded deeply within the sporophytic-tissue layers of ovules and embryo sac. Following the enzymatic maceration, diverse techniques such as agitation, manual micro-dissection, partial enzymatic digestion, prolonged incubation and reincubation in the maceration mixtures have been required for the liberation of FGUs from ovules and the isolated embryo sacs in several plant species (Mól 1986; Wagner et al. 1989; Theunis et al. 1991; Dumas and Mogensen 1993; Katoh et al. 1997; Hoshino et al. 2006), including *P. hybrida* (van Went and Kwee 1990). Alternatively, in this study, we applied an appropriate enzyme formula to the ovules of *P. hybrida* for one-step isolation, without using additional procedures. Egg cell protoplasts and other FGUs were directly emerged and liberated from ovules within 2 hours of enzymatic treatment, expediently. Egg cell protoplasts of *P. hybrida* were clearly distinguished by their different size from other FGUs and by the presence of large vacuole that occupied almost the whole cell, leaving only a small amount of cytoplasm around the nucleus at peripheral position of the cell as shown in our previous study (Sangthong et al. 2009). Since it was difficult to fuse egg cell protoplast (ca. 47 μm) with the average size mesophyll protoplast due to the large vacuole of both type of protoplasts, we selected the small mesophyll protoplast (ca. 25 μm) with less vacuole, as a fusion partner, which resulted in the successful fusion between egg cell protoplast and mesophyll protoplast. We also facilitated the hybridization and hybrid-selection steps by using a micropump-connected microcapillary (Nano Spuit®) for manually collecting each isolated egg and mesophyll protoplasts prior to the fusion of individual pairs, and transferring the heterokaryons into Millicell® plate for partitioning culture surrounded with nurse cells in Petri dish. Therefore, the callus clumps that grew in Millicell® plate could be confident as hybrids. Putting the fusion products around 4–7 cells in the same Millicell® plate seemed to promote cell division and proliferation. Since appropriate distance was kept between each fusant in Millicell®, neither fusion between the fusion products nor mixed growth of different microcolonies occurred during the culture.

Gametosomatic fusion products between different plant lines have been encountered in diverse developmental patterns. In *Brassica*, allotriploid plants could be obtained from the fusants between pollen protoplasts of *B. chinensis* and hypocotyl protoplasts of *B. napus*. However, when changing somatic donor into hypocotyl protoplasts of *B. juncea*, hybrid calli could only differentiate into roots, and no shoots were regenerated (Li et al. 1994; 1996). These reports suggest the importance of the combination of the donor species. In the present study, only fusants between mesophyll protoplast and egg cell from the same *Petunia* line (K2E hybrids) could regenerate and develop into the complete plants. As for gametosomatic fusion between different strains, DKE hybrid callus strains did not show shoot regeneration ability and could only be maintained as callus cultures, whereas CKE and KCE fusants gradually died after developing into microcolonies. Further studies will be needed to clarify the reason for the difficulty in regenerating plants from the gametosomatic fusion between different genotypes of the same species.

Although the expected triploid and an aneuploid hybrid were found in this study, most of the fusion products showed diploid chromosome set. It might be that elimination of the gametophytic genome which occurred in the most fusion products during initial cell divisions without nuclear fusion, resulted in the production of diploid cultures. It is also possible that the aneuploid was produced due to chromosome loss in aberrant mitosis after formation of the hybrid nucleus either during early period of cell division or in a longer period of gradual segregation (Johnson and Veilleux 2001).

In *Petunia parodii*, an autotriploid derived from microspore culture were morphologically superior with a faster growth rate than their diploids and related tetraploids (Gupta 1982). However, the flowers of K2E-1 triploid ($2n=3x=21$) obtained in the present study were obviously smaller and shorter than those of the parent though it possessed the original color. Although the reason for this unexpected change in flower character is not clear but must be clarified by producing more triploid plants with the use of the same as well as different genotypes through the gametosomatic hybridization method established in the present study.

Results of this study demonstrate for the first time that gametosomatic hybrids can be obtained via asymmetric fusion between female gamete and mesophyll protoplast of petunia and that egg cell has the ability to produce gametosomatic hybrid plants (K2E-1 triploid hybrid). Whereas, the hybrids obtained from the fusion between egg and somatic donors of different strains of petunia could not grow properly. Although the reason for the difficulty in regenerating plants from the gametosomatic fusion between different genotypes of the same species is still unclear, it is at least necessary to develop the appropriate medium formula and culture condition, which are applicable to the wide range of intra- and interspecific fusion combinations.

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