Scientia Horticulturae 210 (2016) 143–149

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

# Dynamics of nuclear phase changes during pollen tube growth by using in vitro culture in *Petunia*

Yoichiro Hoshino[a,b,*], Nozomi Eiraku[c], Yoshimi Ohata[b], Fuminori Komai[c]

[a] Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan
[b] Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan
[c] Agri Innovation Center, Saga University, Karatsu, Saga 847-0021, Japan

ARTICLE INFO

*Article history:*
Received 22 May 2016
Received in revised form 16 July 2016
Accepted 18 July 2016
Available online 25 July 2016

*Keywords:*
Bicellular pollen
Generative cell
*Petunia*
Pollen tube
Sperm cell
Vegetative cell

ABSTRACT

In flowering plants, pollen germinates to deliver sperm cells to the egg and central cells during double fertilization. In a bicellular pollen species, sperm cells are formed as the pollen tube grows. However, the process of pollen tube growth is difficult to observe as it proceeds in inside the pistil. Here, an in vitro liquid pollen culture system was applied to pollen of *Petunia* cultivars to analyze the nuclear phase transitions during pollen tube growth by microscopy and flow cytometry. The pollen germination frequencies for eight *Petunia* cultivars were initially evaluated. Among them, the cultivar 'Chiffon' showed the highest pollen germination frequency and was used for further analysis. Fluorescence microscopy revealed that pollen had two nuclei just after germination, indicating the generative and vegetative cells. Twelve hours after pollen germination, three nuclei were observed in the cultures, suggesting that nuclei indicated two sperm cells and one vegetative cell had developed and the generative cells had divided in vitro. Flow cytometry successfully detected nuclear phase changes during pollen tube growth, and the analysis suggested male germ unit formation, which develops from the connection between the generative and vegetative cells, or between the two sperm cells and the vegetative cell. These results show that the pollen grains could germinate in vitro and generative cells could divide to form sperm cells under these culture conditions. This in vitro liquid pollen culture system will shed light on physiological events regulated in pollen tubes.



## 1. Introduction

In flowering plants, pollen delivers the sperm cell required for double fertilization, which occurs between the egg cell and sperm, as well as between the central and secondary sperm cells. Pollen grains released from the male donor anther germinate on the stigma of the female recipient. Germinated pollen grains form pollen tubes that can grow into the pistil and toward female gamete. There are two types of pollen at the mature stage of development: bicellular pollen and tricellular pollen. Bicellular pollen is composed of one generative cell and one vegetative cell in a single grain at the dispersal stage. Tricellular pollen is composed of two sperm cells and one vegetative cell at the dispersal stage. Approximately 70% of flowering plants have bicellular type pollen (Brewbaker 1967; Williams et al., 2014). In bicelluar pollen, two sperm cells are formed from the generative cell in the pollen tube after germination. Cell division in the generative cell forms sperm cells concomitant with pollen germination, followed by pollen tube development. Therefore, the cell cycle of generative cells is thought to be cooperative with pollen tube development. Detecting the timing of these stages of cell division is difficult because the event proceeds inside the pistil of recipient plants. In order to visualize pollen tube growth, Brewbaker and Kwack (1963) developed a pollen culture medium to induce pollen germination and follow pollen tube growth in vitro. The pollen grains of numerous plant species can germinate on this medium (Brewbaker 1967) and direct observation of pollen tube growth has become possible. In our previous study, we improved the culture medium for pollen germination and pollen tube growth (Hirano and Hoshino 2009), and revealed that the addition of yeast extract to the liquid culture medium was effective in improving synchronous pollen germination and increases the pollen tube growth rate. By using the liquid culture medium, the dynamics of nuclear phase changes in pollen tubes were examined with flow cytometry and microscopy

*Abbreviations:* DAPI, 4′,6-diamidino-2-phenylindole; MGU, male germ unit.

* Corresponding author at: Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan.
*E-mail addresses:* hoshino@fsc.hokudai.ac.jp, hoshino@exfarm.agr.hokudai.ac.jp (Y. Hoshino).

http://dx.doi.org/10.1016/j.scienta.2016.07.020
0304-4238/

in *Alstroemeria* (Hirano and Hoshino 2009) and *Cyrtanthus* (Hirano and Hoshino 2010). Furthermore, Hirano et al. (2013) used the liquid pollen culture system to examine DNA damage response after carbon ion beam irradiation.

In the present study, we applied the liquid pollen culture system to *Petunia* cultivars that have bicellular pollen. *Petunia* is an important horticultural crop and its plants have been utilized as potted and garden flowers. During the process of cultivar development, various genotypes among different species have been used for cross-pollination. Therefore, current cultivars are composed of complex genomes that can induce aberrant sexual reproduction. In this study, we examined pollen germination ability by using the liquid pollen culture system in *Petunia* cultivars. The division of the generative cell in the formation of sperm cells in pollen tubes was observed with fluorescence microscopy during pollen tube growth. Moreover, we tried to examine and analyze the nuclear phase changes that indicate generative cell division and male germ unit (MGU) formation by using flow cytometry. MGU describes the physical relationship between generative and vegetative cells, or between two sperm cells and the vegetative cell (Mogensen 1992). In *Petunia hybrida*, MGU was reported in mature pollen and in pollen tubes in vivo (Wagner and Mogensen, 1988). Broothaerts et al. (1991) and Akita et al. (2002) observed *Petunia* pollen germination and successive pollen tube growth by using liquid in vitro culture medium supplemented with polyethylene glycol (PEG). The liquid pollen culture system was applied in *Petunia* for microarray and quantitative PCR analyses to compare gene expression levels between mature pollen grains and pollen tubes (Ishimizu et al., 2010). Dowd et al. (2006) used in vitro pollen tubes to demonstrate that phospholipase has an important role during pollen tube growth in *Petunia*. Suwińska et al. (2015) elucidated that calreticulin is translated on the ribosomes associated with the endoplasmic reticulum by using *Petunia* in vitro pollen tubes. However, previous research has not addressed the nuclear phase changes and the timing of sperm cell formation from generative cells in *Petunia* pollen under in vitro conditions. Here, we investigated *Petunia* sperm cell formation in an in vitro pollen culture system by using fluorescence microscopy and flow cytometry techniques.

## 2. Materials and methods

### 2.1. Plant material

The seeds of *Petunia* cultivars purchased from Takii & Co., Ltd. (Kyoto, Japan) were as follows: *Petunia* F1 Symphony series 'Blue'; *Petunia* F1 Symphony series 'Chiffon'; *Petunia* F1 Symphony series 'Purple'; *Petunia* F1 Symphony series 'Rose Star'; *Petunia* F1 Symphony series 'White'; *Petunia* 'Pirouette Purple'; *Petunia* 'Double Cascade Blue'; *Petunia* 'Double Cascade Mix'; and *Petunia multiflora* F1 Hurrah series 'Blue Veined'. The seeds of these *Petunia* cultivars were sown in a greenhouse and grown under field conditions in the Field Science Center for Northern Biosphere, Hokkaido University, Japan.

### 2.2. Pollen culture in liquid medium for pollen tube growth

Mature anthers were collected just before dehiscence from flowers and kept in a glass Petri dish at room temperature in the laboratory. After dehiscence of the anthers, pollen grains were put in 3.5 cm plastic Petri dishes containing 1.5 ml culture medium. Procedures for pollen culture and for analyzing pollen tubes with flow cytometry were followed according to Hirano and Hoshino (2009).

### 2.3. Evaluation of pollen germination frequency

Pollen germination frequency in each cultivar was evaluated 6 h after pollen culture. Growing pollen tubes were observed with an inverted microscope (Axiovert 200, Zeiss, Oberkochen, Germany). The growing pollen tubes in 5 random microscopic fields were counted under the microscope using a 5× objective lens. Experiments were repeated three times and the data are expressed as the mean of these experiments with standard error calculated.

### 2.4. Microscopic observation of nuclei in the pollen tubes

Pollen tube growth was observed at 1.5 h, 3 h, 6 h, 9 h, 12 h, 18 h, 24 h, and 30 h after pollen culture. The fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) (Bacstain DAPI solution, Dojindo Laboratories, Kumamoto, Japan) was used for staining nuclei in pollen tubes. Before staining, the culture medium containing pollen tubes was transferred to 35 mm-glass bottom dishes (glass surface diameter 27 mm, D110400, Matsunami Glass, Osaka, Japan), and Triton-X 100 was added at a final concentration of 0.5% (v/v) to promote permeability of the DAPI into the pollen tubes. DAPI was then added to the medium at a final concentration of 1 $\mu g\,mL^{-1}$. The treated pollen tubes were observed under an epifluorescence microscope (Axiovert 200, Zeiss, Oberkochen, Germany) with a no. 1 filter set. Images were obtained with a microscope-connected camera (DS-Fi2-L3, Nikon, Tokyo, Japan).

### 2.5. Flow cytometry for nuclear phase changes during pollen tube growth

We designed our experimental procedure according to that developed by Hirano and Hoshino (2009, 2010). Nuclear phases during pollen tube growth were analyzed with a flow cytometer (Ploidy Analyzer PA, Partec, Münster, Germany). More than 5000 nuclei were measured in each experiment.

## 3. Results

Pollen germination and germination frequency were measured in 8 *Petunia* cultivars. Pollen grains started to germinate approximately 1 h–1.5 h after culture. Pollen tubes continued to elongate through the culture period. Some cultivars such as the F1 Symphony series 'Blue' had a low germination frequency. The frequency of pollen germination in each cultivar was evaluated 6 h after liquid pollen culture (Fig. 1). Among them, *Petunia* F1 Symphony series 'Chiffon' showed the highest pollen germination frequency and was used for further experiments.

To determine the behavior of nuclei in the pollen tubes, the nuclei were stained with DAPI 1.5 h, 3 h, 6 h, 9 h, 12 h, 18 h, 24 h, and 30 h after pollen germination. At 1.5 h, while the pollen tubes grew, the nuclei did not move into the pollen tubes (Fig. 2a, b). Two nuclei indicated that the generative and vegetative cells were present in the germinated pollen. At 3 h–9 h, the two nuclei migrated in to the pollen tubes (Fig. 2c–h), but the generative cells had not divided. After 12 h, three nuclei from the two sperm cells and one vegetative cell were observed the majority of the time (Fig. 3a, b). In some pollen tubes at this time point, we did observe only two nuclei from the generative and vegetative cells. Fig. 3c shows an example of a generative cell in the prophase stage preparing for to divide. At 18 h–30 h, pollen tubes were allowed to continue to grow and three nuclei (two sperm cells and one generative cell) were observed in the pollen tubes (Fig. 3d–f).

During pollen tube growth, the connections between the generative and vegetative cells, and those between the vegetative and two sperm cells were observed (Figs. 2 and 3). This suggests that