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# *In Vitro* Culture and Transformation by Agroinfiltration of Lisianthus (*Eustoma russellianus*) Pollen

**Hee Sung Park***

*Department of Plant Biotechnology, Catholic University of Daegu, Gyungsan, Kyunbgbuk 712-702, Korea*

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Optimized conditions for *Agrobacterium*-mediated lisianthus pollen transformation were adjusted using various factors such as temperature, pH and sucrose concentration. Pollen tube growth was successfully achieved in a medium (pollen germination medium; PGM) containing 7-15% sucrose with pH in the range of 5.5-7.0 at temperature of 20-27oC. Lisianthus pollen was vacuum-infiltrated with *Agrobacterium* cell suspension for 20 min, and transformed pollen was confirmed by GUS histochemistry and Southern hybridization following RT-PCR. Transgenic pollen system may be utilized for establishing an area of plant transient expression systems based on the convenient pollen transformation procedure presented in here.

**Key words** – Lisianthus, pollen, pollen germination medium, transformation, GUS gene

Lisianthus (*Eustoma russellianus*), also called eustoma or texas blue bell, is native to the prairies of Texas and Mexico. It has colours including white, purple, cream, pink, pale green and bicolors. In our country, it is one of the major cut flowers and is popularly beloved. Recently, efforts producing modified plants of high values have been done by molecular breeding using pollen. Pollen which contains the male gametophytes and is able to germinate upon pollination and grow through the stigma and style to the ovules to deliver the sperm cells [13,15] can be manipulated as a live vector to transfer a foreign trait to the offsprings. Introduction of the new genetic trait to the pollen can be performed by *in vitro* transformation mediated by *Agrobacterium* or particle bombardment. In fact, this technique has been applied to various plant species including pine, petunia, rice, lily, *Arabidopsis*, wheat and maize [2,3,4,6-8,10,11,16]. For pollen to be transformed, several things should be considered prior to *in vivo* fertilization. These are, for example, proper storage of live pollen for a long term period, optimized conditions for active pollen germination and test of germination inhibition in the presence of certain chemicals including antibiotics that would be treated in the course for optimizing pollen transformation by use of *Agrobacterium* or particle bombardment [12]. In this study in an initial effort to develop pollen as a novel type of plant transient expression system, Lisianthus pollen was examined for its germination and elongation under conditions varied of temperature, pH, chemicals. Transformed pollen using *Agrobacterium* cells harboring GUS DNA via vacuum infiltration was evaluated by histochemistry and RT-PCR.

***Corresponding author**
Tel : +82-53-850-3245, Fax : +82-53-850-3459
E-mail : hspark@cu.ac.kr

## Materials and Methods

### *In vitro* pollen growth

Lisianthus (*Eustoma russellianus*) with single-formed and purple-coloured flower was purchased from the local market. Its pollen grains after dehiscence were manually collected and immediately tested or stored until use in a deep freezer (-70°C). Pollen germination medium (PGM) contained basically 1.6 mM $H_3BO_3$, 1.8 mM $Ca(NO_3)_2$ and 15% sucrose [5]. For germination, pollen grains were placed in PGM in the dark for about 24 h.

### Pollen growth measurement

Germinated and elongated pollen samples in PGM were poured onto a steel sieve (120 mesh) and the retained samples were immediately blotted on paper towel for measuring fresh weight.

### Agrobacterium-mediated pollen transformation

Plant expression vector pBI121 which contains GUS reporter gene (*uidA*) was introduced to *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method [1]. For transformation, the overnight-grown *Agrobacterium* cells

were resuspended in PGM, added to pollen grains in PGM and infiltrated for 30 min in a vacuum chamber connected to an aspirator (ASP-13, Iwaki Glass Co.) [16]. After this, pollen grains were further incubated for 24 h at 27°C in the dark.

### Histochemistry

A histochemical GUS enzyme assay for pollen was performed basically as described [9]. Pollen samples were fixed in 0.3% formaldehyde solution containing 10 mM MES (pH 5.6) and 0.3 M mannitol for 45 min at room temperature, placed in a vacuum container for 1 min and then washed three times with 50 mM $NaPO_4$ (pH 7.0) solution. Treated samples were immersed in 50 mM $NaPO_4$ (pH 7.0) solution containing 0.1% X-Gluc and 1% dimethylformamide (v/v), incubated at 37°C until blue color appeared and then transferred to 70% ethanol.

### RT-PCR and Southern hybridization

Total RNAs were isolated by the guanidine isothiocyanate extraction method [13], treated with RNase-free DNase (Promega) and used to synthesize 1st strand cDNA by M-MLV reverse transcriptase for 1 h at 42°C using oligo $(dT)_{15}$ primer. Following this, gradient PCR was performed using a set of primers [a sense primer including ATG, 5-CGTAATTATGCGGGCAAC-3 and the oligo $(dT)_{15}$] by 30 cycles of reactions: 94°C, 30 sec; 45-58°C, 30 sec; 72°C, 90 sec. PCR products separated on 1% agarose gel was transferred to nytran membrane by downward transfer method using alkaline buffer (1 M NaCl, 400 mM NaOH) and fixed by using UV-crosslinker (CL-1000, UVP Inc. USA). Hybridization [buffer (5XSSC, 0.1% SDS, 5% dextran sulfate), 60°C, 16 h] was performed using 1.7 kb GUS DNA probe labelled by Bright Star Psoralen-Biotin nonisotopic labeling kit (Ambion Inc. USA), and the result was detected by autoradiography using CDP-Star detection system (Ambion Inc. USA).

## Results and Discussions

### Effect of Sucrose on pollen growth

Generally, 50-100 mg of pollen grains were used for incubation in 20 ml of PGM in a 80 mM culture dish. The effect of sucrose supplement (1-20% w/v in PGM) on pollen growth was examined after 24 hr incubation at 27°C in dark condition. Most of the pollen species examined so far have been reported to need sucrose supplement in the range of 7-20% for their normal pollen growth *in vitro*. In this study, lisianthus pollen germination was monitored to less satisfactory by lower sucrose content (1-3%) or highest (20%) than medium sucrose content (5, 7, 10, 13 or 15%) from both microscopic observation and wet fresh weight measurement (Fig. 1). The pollen tube from PGM containing 20% sucrose is shown to be much shorter than that from 15% sucrose. In fact, not all of pollen grains germinated in medium supplemented with the medium sucrose content but only 20-30%, representing genetical, environmental or physiological hindrance. Also, fungal contamination may be functioning as a negative factor for pollen germination because long incubation more than two days usually resulted in fungal growth in the medium.

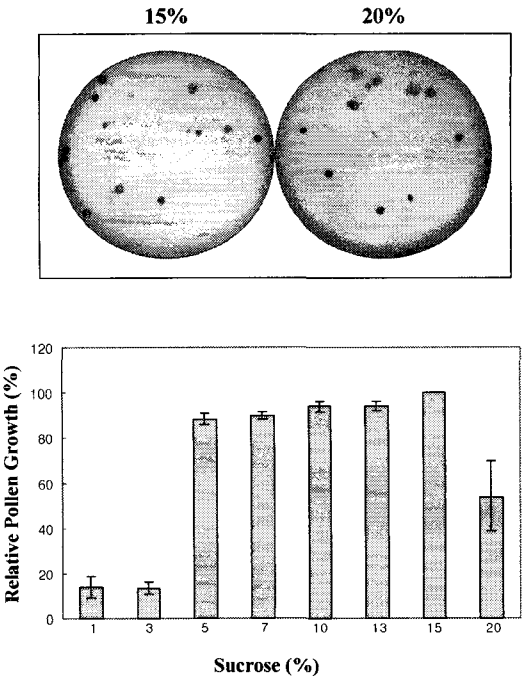


Fig. 1. Effect of sucrose supplement on pollen germination and growth. Upper panel: pollen growth (100x magnified view) supplemented with 15% and 20% sucrose in PGM. Lower panel: Pollen growth in the presence of 1-20% sucrose in PGM was measured by weighing samples retained on 120 steel mesh from pollen liquid culture. Pollen growth in PGM containing 15% sucrose was adjusted as 100% for comparison with those in PGM containing other sucrose contents. The relative pollen growth means average value obtained from four independent experiments.

### Effects of pH, temperature and kanamycin on pollen growth

Meanwhile, in PGM containing 15% sucrose, changes of overall pollen morphology was not observed in pH of 5.0-7.5 or at temperature of 19-27°C (data not shown). So, optimal germination for lisianthus pollen *in vitro* could be achieved in PGM with 15% sucrose at pH 5.5-6.0 during incubation at 22-27°C in darkness. Besides these factors influencing lisianthus pollen growth *in vitro*, antibiotics should be examined for toxicity to pollen growth for the purpose of appropriate selection of the transgenic host. Antibiotic such as kanamycin (km) would be added following plant transformation procedure. Prior to this selection, km toxicity to normal lisianthus pollen germination and growth was tested at the level of 0-200 mg/l in the optimized PGM (15% sucrose, pH 5.7) at 27°C for 24 h. Unexpectedly as seen in Fig 2, even 125 mg/l km treatment didn't completely inhibit pollen germination and growth, implying high resistance to this antibiotic in comparison to the typical toxic level of 50-100 mg/l for the most plant species. By treating km more than 125 mg/l, pollen growth, however, was highly impaired; germination inhibition, pollen tube shortening and a burst. Tomato and tobacco pollen grains were reported to be more sensitive. In another antibiotics treatment, cefotaxime at 125-250 mg/l for eradicating residual Agrobacterium following transformation did not harm the pollen growth.

### GUS expression in lisianthus pollen

About 100 mg of lisianthus pollen grains dispersed in 5 ml of the optimized PGM containing 15% sucrose were infiltrated with *A. tumefaciens* LBA4404 harboring pBI121 for 20 min and then new 15 ml PGM was added for 16 h to full pollen growth. Following this, km (125 mg/l) and cefotaxime (250 mg/l) were treated for 8 h to the transformed pollen for selection and *Agrobacterium* removement, respectively, as described above. Transformed pollen transformation was visualized by histochemical staining resulted from GUS enzyme activity on chromogenic X-Gluc substrate. In Fig. 3, some of pollen grains or pollen tubes showed dark blue color in contrast to the others with almost translucent color. In nontransformed samples, all of them did not clearly show color stain. These results suggest that GUS enzyme expression occurs in transformed lisianthus pollen. In transgenic sample, pollen grains which did not germinate but still display color stain may be due to normal cellular activity of GUS enzyme even in deterred pollen germination and elongation procedure. The transgenic pollen confirmed by histochemistry was further analyzed by RT-PCR amplification of pollen mRNA transcripts. 1st cDNA synthesized using oligo ($dT_{15}$) as a primer was amplified by PCR set at gradient annealing temperatures of 45-58°C, and then the resulting products were compared to nontransgenic pollen. Prior to this, DNA contamination possibly derived GUS expression vector from recombinant *Agrobacterium* was eliminated by cefotaxime treatment following pollen growth. This, in deed, was ensured by no occurrence of bacterial colony formation on LBagar plate. Further steps of extensive pollen culture washing and DNase treatment to the total RNA preparation were done for the same purpose. The amplified PCR product from each of the gradient annealing temperature is shown in Fig. 4A. Multiple DNA products are seen from both nontransgenic and transgenic samples. All of these DNA bands were analyzed by Southern blot at stringent

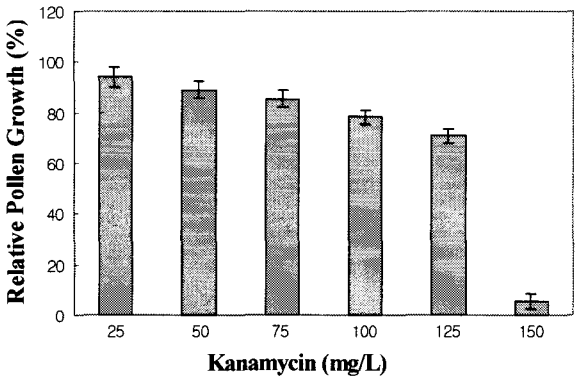


Fig. 2. Effect of kanamycin on pollen growth. Pollen growth in PGM without km was adjusted as 100% for comparison with those in PGM containing km of different concentrations. The relative pollen growth means average value obtained from four independent experiments.

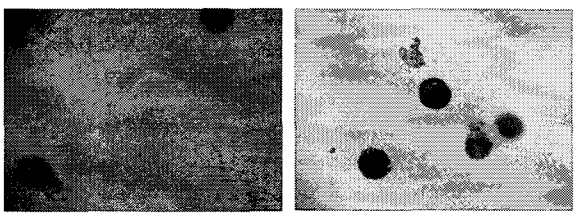

Fig. 3. GUS histochemistry of transgenic lisianthus pollen. Blue color spot owing to GUS expression appears either in pollen grains (right panel) or pollen tube (left panel). Untransformed pollen remains yellow.

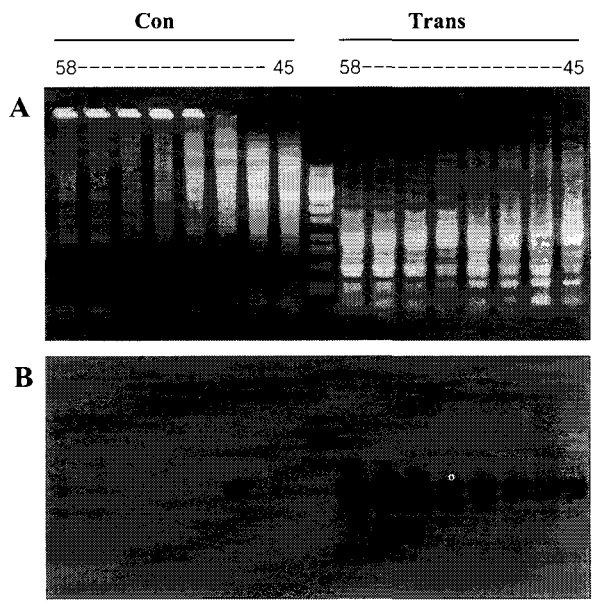


Fig. 4. RT-PCR analysis of GUS mRNA from transgenic pollen samples. A: Agarose gel electrophoresis of RT-PCR products from nontransgenic (Con) and transgenic (Trans). Each lane represents products from PCR reaction set at annealing temperature in the range of 58-45oC in a gradient mode. The center lane displays DNA size marker. B: Result of Southern blot hybridization for PCR products shown in (A). For this, temperature was set at 60oC during hybridization for 16 h.

hybridization temperature (60°C) and the results are shown in Fig 4B. There is clearly a band corresponding to 1.8 kb from the transgenic but not from the nontransgenic, implying a stable GUS mRNA expression.

In plant system, transient expression has been successfully practiced with ease and convenience using cells, tissues and whole bodies with the aid of *Agrobacterium*, bombardment or electroporation since plant transformation techniques devised [6]. In this study, transient expression using lisianthus pollen was evaluated after *in vitro* pollen growth and transformation via agroinfiltration. The results obtained here may be suggested as a preliminary effort to develop the pollen as one of the practicable plant systems for transient expression in the future.

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## 초록 : Lisianthus 화분의 기내배양 및 Agroinfiltration에 의한 형질전환

박희성*
(대구가톨릭대학교 식물유전공학과)

*Agrobacterium*을 이용한 lisianthus화분의 형질전환을 위하여 적정조건을 수립하였다. 화분의 발아 및 화분관의 발달은 화분발아배지(pollen germination medium; PGM)에 sucrose를 7-15% 첨가 시키고 pH조건을 5.5-7.0으로 조절하여 20℃-27℃에서 배양할 경우 성공적으로 이루어졌다. 형질전환을 위하여 *Agrobacterium* 현탁액을 lisianthus화분배양액에 첨가하여 진공침윤을 20 min 실시하였으며 형질전환화분은 조직화학적 분석 그리고 발현되는 GUS mRNA를 이용한 RT-PCR 및 Southern hybridization에 의한 DNA산물 분석 등에 의하여 GUS발현을 확인하였다. 이러한 결과를 통하여 화분을 이용한 일시발현기술을 제시하게 되었다.