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Lily Pollen Growth *In vitro* and *Agrobacterium*-mediated GUS Gene Transformation via Vacuum-Infiltration

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Abstract

Conditions for lily pollen growth *in vitro* and transformation were optimized. Active pollen tube development was achieved effectively in a medium containing 7% sucrose with pH adjusted to 5.7 at the temperature of 27℃ for about 16-24 hours. Pollen growth was little impaired by the presence of kanamycin at concentration up to 100 mg/L. Pollen rains near the beginning of germination stage were more reliable for *Agrobacterium*-mediated GUS DNA transformation via vacuum infiltration lasted for 20-40 minutes. GUS DNA integration and its expression in fully developed pollen tubes could be confirmed by Southern blot hybridization, RT-PCR and histochemical staining.

Introduction

In nature, pollen grain which contains the male gametophytes can grow its tube upon pollination through the stigma and style to the ovules to deliver the sperm cells (McCormick, 1993; Taylor and Helper, 1997). In efforts to construct desirable progenies of certain plant species, the pollen may be manipulated so as to be introduced a foreign gene in vitro by *Agrobacterium*- or particle bombardment-mediated transformation that has been commonly performed to the explants. So, transgenic pollen has been exploited as a live vector to transfer a new gene to the egg for the purpose of molecular plant breeding as shown with various plant species (Aroman et al., 1997; Bethtold et al., 1993; Fernando et al., 2000; Haggman et al., 1997; Hess and Nimmrichter, 1990; Langridge et al., 1992; Luo and Wu, 1989; Tjokrokusumo et al., 2000). To verify the transgenesis in these performances, pollen grains sometimes are needed first to be grown *in vitro* but with a great ease, that is, rapid germination and elongation in a simple medium with only several nutrients (sucrose, boric acid and calcium and one or two chemicals in addition when required) at moderate temperature within a day or two. Taken such feasible facts of pollen transformation and manipulation, it may be assumed that the pollen could be employed as a disposable host for a variety of foreign gene expression to be confirmed in a rapid mode. So in this study, lily pollen grains produced in a large quantity in nature were chosen to establish an optimized pollen tube growth *in vitro*, transformation and gene expression by testing differed culture conditions in respects of sucrose content, temperature and pH. *Agrobacterium*-mediated transformation was performed via vacuum infiltration for varied times to the pollen grains at different growing stages. The results were analyzed by cellular and molecular techniques.

Materials and Methods

Pollen grains and growth *in vitro*

Recently dehisced anthers were detached from flowers of lily (*Lilium longiflorum*) and their pollen grains were collected by passing through a steel sieve device. They were immediately used or stored in a deep freezer. The lily pollen growth media (PGM) was composed of 1.6 mM H_3BO_3, 1.8 mM $Ca(NO_3)_2$ and sucrose (Fernando et al., 2000). Pollen growth *in vitro* was attained by incubating in PGM in the dark for 16-24 hrs. Fully elongated pollen percentage was measured from five replicates each with at

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least 300 pollen grains counted under a microscope.

Pollen transformation

Plant expression vector pBI121 which contains GUS reporter gene (*uidA*) was introduced to *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method (An et al., 1988). For transformation, pollen grains were mixed with the overnight-grown *Agrobacterium* cells resuspended in PGM and placed in a closed chamber for vacuum operation with an aspirator (ASP-13, Iwaki Glass Co.) (Tjokrokusumo, 2000). Vacuum-infiltrated pollen grains were washed several times with sterile water and resuspended in PGM containing kanamycin for further incubation of 16 to 24 hrs in the dark.

Southern hybridization and RT-PCR

Pollen samples were ground to a fine powder in the presence of liquid nitrogen using mortar and pestle and extracted to isolate genomic DNA according to the CTAB/chloroform extraction protocol (Taylor et al., 1993). Genomic DNA which was digested with *Xba*I and *Sac*I, electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Hybond N, Amersham) was hybridized to the 1.8 kb GUS DNA fragment excised from pBI121 plasmid by *Xba*I and *Sac*I. Labeling was according to the ECL random prime system (Pharmacia). Hybridization was performed at 50°C for 24 hrs in buffer containing 5x SSC, 0.1% SDS, 5% dextran sulfate, 100 μg/ml denatured sheared salmon sperm DNA and 5% Pharmacia liquid block. Results were monitored by ECL detection system (Pharmacia) following anti-fluorescein-HRP reaction. For detecting GUS gene expression, total RNAs which were isolated by the guanidine isothiocyanate extraction method (Strommer et al., 1993) and treated with RNase-free DNase (Promega) were used to synthesize 1st strand cDNA by MMLV reverse transcriptase for 1 hr at 42°C using a GUS-specific antisense primer (5-TTTGTCACGCG CTATCAG-3) representing a region at 1 kb downstream from ATG. Following this, PCR was performed using a set of primers (a sense primer including ATG, 5-CGTAAT-TATGCGGGCAAC-3 and the antisense sequence described above) by 30 cycles of reactions: 94°C, 30 sec; 55°C, 30 sec; 72°C, 90 sec.

Histochemical staining

A histochemical GUS enzyme assay in the transformed pollen was performed basically as described by Jefferson (1987). Pollen tubes 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. They 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 came into a view and then transferred to 70% ethanol.

Results and Discussion

The effects of sucrose, pH and temperature on pollen tube growth *in vitro*

In general for a proper spacing of the elongated pollen tubes, 100 mg of pollen grains were used for incubation in 20 mL of PGM in a 80 mm culture dish. At 27°C in the dark, varied sucrose content (1, 3, 5, 7 and 10%) in PGM was compared for pollen growth. Only about 30-50% of pollen grains showed a full tube growth upon the supplement of 1, 3 or 5% of sucrose. But, the figure increased to 70-95% by 7 or 10% sucrose. Pollen growth in 7% sucrose PGM was even more stably performed when incubated at 27°C than at the other temperatures tested in the range of 20-30°C (Figure 1). The pH in the range of 5.0 to 6.0 was also examined to get a more reliable consequence of pollen growth at pH adjusted to 5.7.

Pollen transformation via vacuum infiltration

Pollen grains were transformed using *A. tumefaciens* harboring pBI121 via vacuum infiltration. Pollen grains at different growth stage were comparatively tested in respect of the transformation efficiency to be determined by the extent of histochemical staining. For this, the vacuum infiltration-aided transformation process was carried

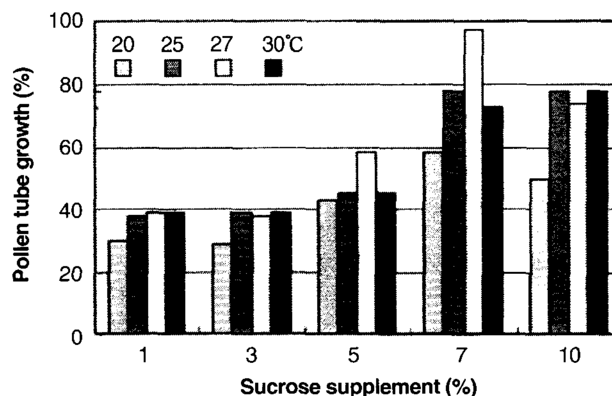

Figure 1. The effects of sucrose and temperature on pollen tube growth *in vitro*

out for 20 min at room temperature and then the pollen samples were continued to the full growth. Pollen grains at the beginning of germination (PBG) with a small bud emerged were more reliable than those prior to germination or with tubes elongated to a medium length. Usually, germination occurred 1-2 hrs following incubation in PGM (Figure 2). To minimize the accompanying *Agrobacterium* cells following transformation process, washing was done with sterile water or PGM several times. To the PBG, vacuum treatment for 5, 10, 20, 40, 60 or 120 min was compared. As a result, 20 or 40 min process was more dependable (data not shown).

GUS gene transfer and mRNA synthesis

Lily pollen grains were transformed and, therein, GUS DNA integration and mRNA synthesis were analyzed. Genomic DNA was extracted from fully matured pollen tubes and digested with. *Xba*I and *Sac*I for Southern blot hybridization. The results are represented in Figure 3. A DNA fragment at the size of 1.8 kb was evident from the transformed but none from the untransformed, strongly indicating an integration of GUS DNA into the pollen chromosomal DNA. This, however, did not mean its identity in the nucleus whether tube or germ cell. The mRNA synthesis from the transformed pollen tube was analyzed by PCR following reverse transcription of total RNA as a template. The amplified DNA product of PCR was detected in the agarose gel at the site corresponding to 1 kb in length. The size of PCR product was due to the primers designed so as described in materials and methods. This PCR product was further confirmed by gene-cleaning and digestion with *Bal*I to produce two DNA products with similar size as shown in Figure 4.

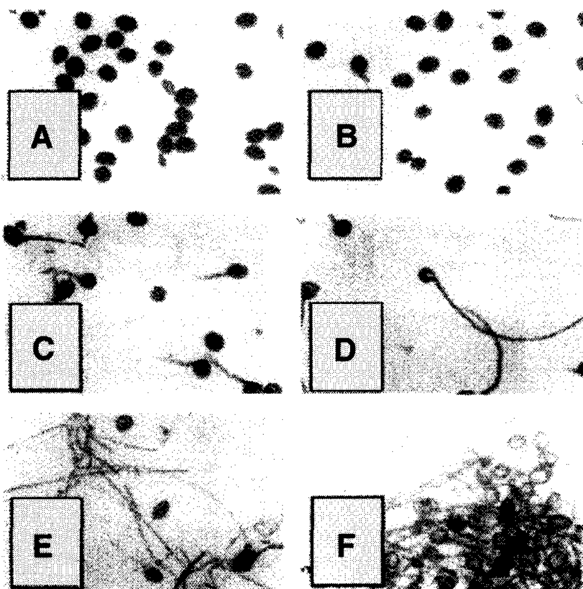

Figure 2. Pollen germination and tube growth *in vitro* during a culture period. Marked A, B, C, D, e and f represent 0, 3, 5, 7, 10, 16 hours, respectively.

Kanamycin (km) selection and GUS expression

For the purpose of selective harvest of transformed

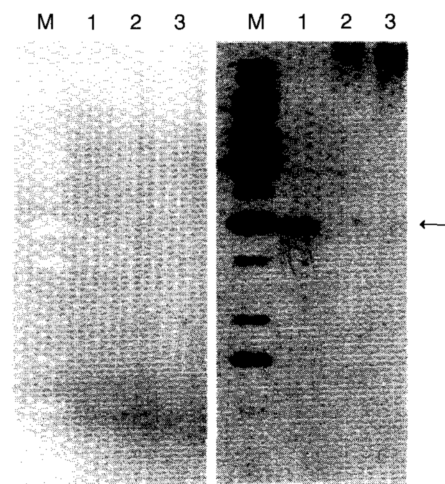

Figure 3. Gel electrophoresis pollen DNA and Southern hybridization. M, DNA size marker; 1, GUS control DNA; 2 and 3 are *Xba*I and *Sac*II-digested DNA from the control pollen and the transformed, respectively. 1′, 2′ and 3′ are the results of Southern hybridization for lanes 1, 2 and 3, respectively. The arrow indicates the GUS DNA in the transformed pollen.

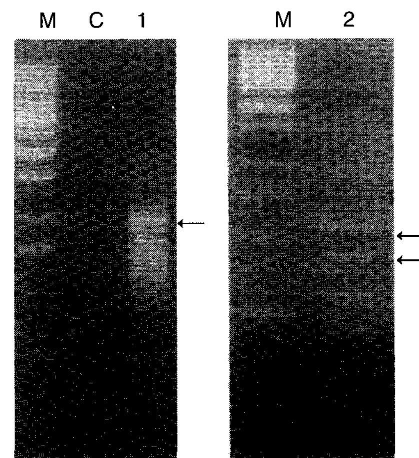

Figure 4. PCR of reversely transcribed pollen mRNA. M, DNA size marker; C and 1, PCR product of the control and the transformed pollen, respectively; 2 *Bal*I, digest of PCR product form the transformed. The arrow in the left panel indicates 1 kb DNA product and the arrows in the right are the enzyme-digested.

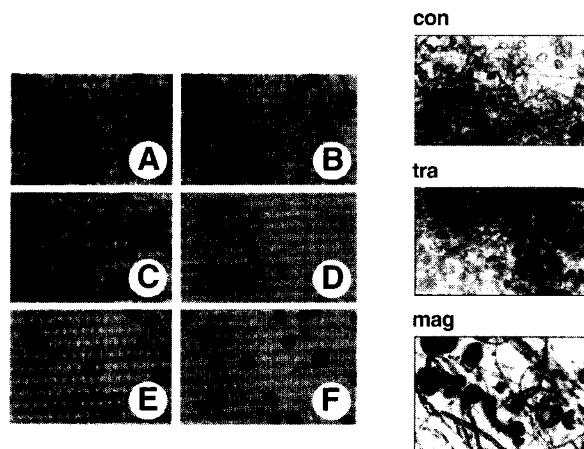

Figure 5. Lily pollen sensitivity to kanamycin and histochemical staining. Left panel: Boxes a, b, c, d, e and f represents km treatment at the concentrations 50, 100, 125, 150, 175 and 200 mg/L, respectively, in the pollen trowth media. Right panel: histochemical staining of the control pollen (con), the pBI121 transfomed (tra) and its magnified view (mag).

pollens resistant to km, control pollen grains were challenged to km from none to 200 μg/mL included in the optimized PGM (7% sucrose, pH 5.7) at 27°C for 24 hrs. The presence of km up to 100 μg/mL did not seem to inhibit a normal tube growth (Figure 5). With km above this concentration, pollen growth, however, was strongly sensitized; that is, germination inhibition, pollen tube shortening and a burst. Tomato and tobacco pollen grains were reported to be more sensitive; usually 10-20% were inhibited at 50-100 μg/mL of km in a gradual manner (Chesnokov and Manteuffel, 2000). Fully elongated pollen tubes were fixed for reaction with a GUS substrate, X-Gluc. In the transformed, blue colors streamed along the full path of the pollen tube, at least suggesting the translated product in the cytoplasm instructed by GUS DNA from the tube cell nucleus. The control did not show the color occurrence inside under microscopic observation. Meanwhile, almost all of the transformed pollens grown in either the presence or absence of km (100 μg/mL) showed almost an equal demonstration of the GUS activity implying a successful transgenesis of the most of pollen population by the vacuum infiltration method.

Acknowledgement

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