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Transient Expression of β-glucuronidase(GUS) gene in Immature Ovules and Calli Derived from Cottonwood Species (*Populus deltoides*) by Microprojectile Bombardment[1]

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포플러의 未成熟 胚와 캘러스에서 遺傳子銃에 依한 GUS-gene의 一時的 發現[1]

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ABSTRACT

Excised immature ovules and calli derived from the stems of cottonwood were bombarded with microprojectiles carrying plasmid DNA containing CaMV-35S promoter and β-glucuronidase(GUS) gene. After bombarded, the expression of GUS gene was detected by the assay of 5-bromo-4-chloro-3-indolyl-β-gluconide(X-gluc). Transient gene expression was measured by counting the number of distinct regions of GUS activity per explant. As major parameters, the number of shots and the period of exposure to X-gluc after the bombardment were investigated for detecting GUS gene expression. In this experiment, the percents of GUS gene expression showing spots were 56.8 from immature ovules and 75.9 from micro-calli of cottonwood species. Among the treatments, two consecutive shots and 48 hour exposure produced about 25.75±2.77(per ovule), 11.43±1.22(per mini petridish) spots, respectively. Microprojectile particle bombardment provides a useful method to assay transient expression in both types of explants. Furthermore, our results represent that the excised ovule and/or the calli might be stably transformed by the biolistics.

Key words : *Particle bombardment, transient gene expression,* GUS *gene expression, Populus, immature ovule, calli*

要 約

미류나무의 미성숙 ovule과 줄기로부터 유기된 캘러스에 plasmid pBI221 유전자를 유전자총을 이용하여 인위적으로 삽입하였다. Plasmid pBI221은 CaMV-35S 유전자에 의하여 발현되는 β-glucuronidase(GUS) reporter 유전자를 포함하고 있다. Plasmid pBI221이 물리적으로 삽입된 후 GUS 유전자의 발현정도는 5-bromo-4-chloro-3-indolyl-β-gluconide(X-gluc)의 반응에 의해 분석되었고, 유전자의 일시적 발현현상은 ovule, 캘러스 시료에 X-gluc substrate의 반응에 의하여 나타나는 뚜렷한 점(spot)의 수에 따라서 조사하였다. 본 실험에서 particle bombardment후 GUS유전자의 발현검정에

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있어 가장 중요한 요인은 bombardment 횟수와 X-gluc substrate에 노출된 시간이었다. X-gluc substrate와의 반응결과, 캘러스와 ovule에서 각각 56.8%, 75.9%의 반응 점들을 나타냈다. 여러 처리중 두번의 연속적인 shot와 bombardment 이후, X-gluc과 sample을 48시간 반응시킨 후 24시간 동안 alcohol로의 침지가 가장 많은 수의 점을 유기시켰고, 이들 반응으로부터 평균 25.75±2.77(ovule), 11.43±1.22(calli)개의 반응 점을 보였다. 유전자총에 의한 외래 유전자 도입에 관한 본 연구는 두종류의 시료로부터 빠른 시간 내에 유전자 발현을 볼 수 있을 뿐만 아니라, 지금까지 *Agrobacterium*을 이용한 형질전환이 보고되지 않은 미류나무(*Populus deltoides*)의 형질전환 연구에 대체 방법을 제공하리라 생각된다.

INTRODUCTION

The abilities to express foreign genes in plant cells are important in genetic engineering of tree species in biotechnology program. This primary step is necessary to understand gene expression and regulation as well as to produce transgenic plantlets. Using an indirect system, genetic transformation of poplar species has been successfully established by co-culture systems with *Agrobacterium tumefaciens* or *A. rhizogenes*(Block, 1990 ; Brasileiro et al., 1991 ; Chun et al., 1988 ; Confalonieri et al., 1994 ; Fillatti et al., 1987 ; Heuchelin et al., 1991 ; Hollick and Gordon, 1993 ; Klopfenstein et al., 1993 ; Leple et al., 1992 ; McCown et al., 1991 ; Strauss et al., 1994). However, the use of co-cultivation has been limited only to dicots because of the difficulties of plant regeneration from those plant tissues. Microprojectile bombardment system has been demonstrated as a reliable method for a wide range of organisms such as animals, algae, yeasts, and monocot plants and of organelles such as mitochondria and chloroplasts(Boynton et al., 1988 ; Johnston et al., 1988 ; Klein et al., 1992). This system has been successfully recovered genetic transformation of corn, rice, wheat, and soybean among major crop species(Cao et al., 1992 ; Chibbar et al., 1991 ; Li et al., 1993 ; McCabe et al., 1988 ; Songstad et al., 1993 ; Takeuchi et al., 1992) and several woody species(Charest et al., 1993 ; Goldfarb et al., 1991 ; Loopstra et al., 1992 ; McCown et al., 1991 ; Stomp et al., 1991 ; Wilde et al., 1992).

The genus *Populus* has many advantages as a model system for biotechnological research with trees because it contains a wide genetic diversity and hybridizes easily between species(Ahuja, 1984 ; Hall et al., 1990 ; Herrmann and Seuthe, 1982). Recently, plant biotechnology has been advanced in tree improvement programs. Nevertheless, most researches have been concentrated on tissue culture with tree species(Agrawal and Gupta, 1991 ; Coleman and Ernst, 1990 ; Kang and Hall, 1996a ; 1996c ; Nadel et al., 1992 ; Son and Hall, 1990). Among *Populus* species, most biotechnological researches have been conducted with aspen species or cottonwood hybrids since they are easy to be established in *in vitro* conditions. In the case of *Populus deltoides*, the lack of regeneration system from leaf discs has been the major obstacle when *Agrobacterium*-mediated transformation is to be applied.

In present research, we demonstrate the transient expression of a reporter gene for β-glucuronidase(GUS) in immature ovules and microcalli of cottonwood(*Populus deltoides*). This paper is the first report for genetic transformation via particle bombardment with explants of excised ovules in pure poplar(*Populus deltoides*). Our results suggest that the biolistics application can be possible for stable genetic transformation for tree improvement program of pure cottonwood species.

MATERIALS AND METHODS

Plant Materials

Immature ovules

Poplar breeding program was cooperated with Iowa State University, University of Washington, and Boise Cascade Company in the United State. To hybridize the cottonwood species, male and female flower buds were collected in the early

January around mid west regions ; Iowa, Illinois, Minnesota, and Missouri and some pollen sources were obtained from University of Washington. Pollinations were conducted in Forestry greenhouse at Iowa State University. Because of the embryo abortion during hybridization, immature ovules were rescued before they abort(Kang and Hall, 1996b). Capsules were collected 14 days after they reached stigma receptivity and collection was repeated at days 20, 26, and 32. The capsules were sterilized with 70% ethanol for 1 min, followed by 20% Clorox for 20 min, and then rinsed four times with distilled water. For plant regeneration as a preliminary study, immature ovules were isolated aseptically from the capsules and placed onto the surface of 20mℓ of WPM(Woody Plant Medium) containing different types and various concentrations of PGRs(Plant Growth Regulators). The cytokinins, BA(6-Benzylaminopurine), zeatin, kinetin, and 2-iP(6-Dimethylallylaminopurine), were used at the concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0mg/L. The regeneration system was followed by the modification of poplar ovule culture system(Kang and Hall, 1996b). The isolated ovules were used for biolistics.

Callus induction

Nodal segments(3 to 4cm in length) containing axillary buds were collected from two-month-old stock plants of cottonwood(*Populus deltoides*, IS-31 clone), that were maintained under greenhouse conditions. The explants were obtained from the top branches of stock plants with succulent tissues. The collected nodal segments were immersed in distilled water for 3 hours at room temperature, dipped in 70% ethanol for 1 min, sterilized in a solution of 2% sodium hypochlorite for 40 min, and then rinsed 4 times with sterilized deionized water. Callus induction was conducted on WPM supplemented with 2.0mg/L of 2,4-D(2,4-Dichlorophenoxyacetic acid) at 25℃ under the dark condition. The induced calli were proliferated on subculture medium containing the same concentrations of Plant Growth Regulators.

Bacterial Strain and Plasmid Preparation

E. coli strain harboring plasmid, pBI221 was the generous gift from Dr. Loren C. Stephens at the Department of Horticulture, Iowa State University, USA. Plasmid pBI221 contains a β-glucuronidase(GUS) gene as a reporter regulated by the 35S cauliflower mosaic virus promoter. The 3.0kb *Hind*III-*Eco*RI fragment of pBI121 was cloned into the corresponding sites of pUC19 (2.7kb) to create pBI221 plasmid. The strain was cultured overnight on LB liquid medium at 37℃. The plasmids were extracted by using the Mini Plasmid Preparation method(Promega Technical Bulletin).

Plasmid pBI221 containing β-glucuronidase gene was isolated from *E. coli* DH5α strain. The plasmid map was represented in Fig. 2. Plasmid DNAs were digested and electrophoresed to determine the purity. Fig. 3 showed clear banding patterns on 1.5% agarose gel. It was represented that the plasmid map was constructed with the digestion of *Eco*RI and/or *Hind*III restriction enzymes(Fig. 3).

Tungsten and DNA Preparation

GE tungsten 1.1μm(60mg) was put into 15mℓ centrifuge tube and 2mℓ 0.1M HNO_3 was added and then the mixture was sonicated on ice for 20 min. After withdrawing HNO_3, 1mℓ sterile deionized water was supplemented and then samples were transferred to a 2mℓ Sarsfedt tube. The pellet particles were centrifuged with 15,000 RPM for 30 min. After removing deionized water, 100% ethanol was added and then centrifuged. The ethanol was replaced to 1mℓ sterile deionized water and the sample was divided into four aliquots in 2mℓ tubes, and added 750μℓ of sterile deionized water to each tube. The prepared sample was frozen at -80℃. To prepare plasmid DNA, 50μℓ tungsten suspension was aliquoted into 1.5mℓ tube containing 10ug DNA, 50μℓ, 2.5M $CaCl_2$, and 20μℓ 0.1M spermidine. The mixture was incubated for 10 min at room temperature. The mixture was then centrifuged for 10 sec at 10,000 RPM. Finally, a 10μℓ tungsten aliquot was used per shot. DNA delivery into the immature ovules and calli induced from the

stems of cottonwood was carried out using a DuPont Biolistic Particle Delivery System(PDS) 1,000, with a vacuum pressure following manufacturer's recommendations. Bombardments were conducted at the presence of 1,100 PSI rupture disk with immature ovules and micro calli. Bombardment system was demonstrated schematically for the samples(Fig. 4).

Determination of GUS-activity

After bombardment, the calli were grown at 25℃ in the dark and immature ovules were maintained in the light condition for 1 to 3 days. These samples were placed in microtitre plates containing GUS assay buffer of 0.5mg/mℓ concentration. The GUS assay buffer contained 1.0M $NaPO_4$(pH 6.8), 0.1M $K_3Fe(CN)_6$, 0.1M $K_4Fe(CN)_6$ H_2O, 0.5M EDTA, 10% Triton X-100, and 100mg X-gluc. After the infiltration of the buffer, the explants were immersed in 70% EtOH for 30 min and were finally examined under Leica Wild M10 Stereo Microscope.

RESULTS AND DISCUSSION

Sample Preparation

In a preliminary test of poplar breeding program, the hybridization experiments were conducted with the various sources of male and female plants(Fig. 1-A). After 2 weeks of culture, the explants of immature ovules on shoot proliferation medium produced multiple shoots. Shoot formation was significantly affected by the types and the concentrations of plant growth regulators supplemented in the medium and the degree of embryo maturation(no data present). Even though the shoots were mainly produced from immature embryos, tiny shoots were also produced at the ends of expanding cotyledons(Fig. 1-B). Among the developmental stages of immature ovules within a single capsule, twenty-day old ovules after pollination were the best explants for shoot proliferation on WPM(Woody Plant Medium) supplemented with a higher concentration(5.0mg/L)

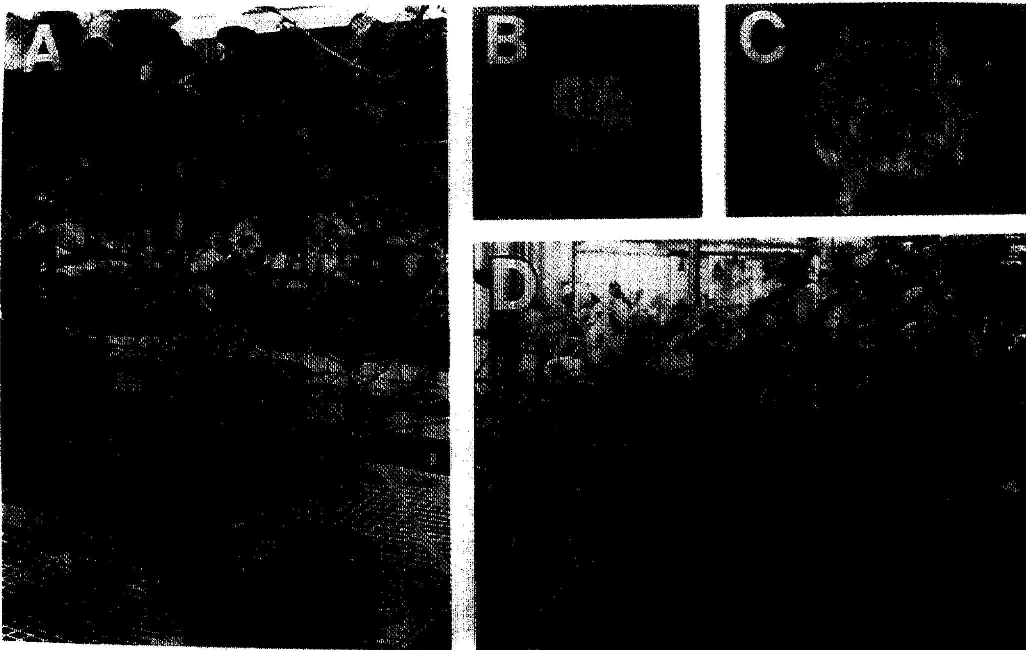

Fig. 1. Shoot formation from immature ovules in poplar breeding program.
(1)Hybridization of *Populus deltoides* in greenhouse, (2)Embryo rescue from shoot multiplication of immature ovule, (3)Shoot elongation of multiple shoots in WPM containing 0.02mg/L NAA, (4)Plants after transplanting to soil mix and six weeks of growth in greenhouse.

of zeatin. Shoots regenerated from immature embryos were excised and transferred to the elongation medium containing a half strength WPM supplemented with 0.02mg/L IBA(Fig. 1-C). After 2 months subculture, the plantlets fully elongated in *in vitro* conditions. Finally, the expanded plantlets were transferred to cell trays containing an artificial soil mixture(vermiculite : perlite : peat=1 : 1 : 1 by volume) in a shaded mist bench under greenhouse conditions. After 2 weeks, these plantlets were moved to a common bench. Among them, more than 97% of the plantlets survived(Fig. 1-D).

Calli were initiated from stem culture of cottonwood on WPM supplemented with 2.0mg/L of 2,4-D in 25℃ under the dark condition. The induced calli were cultured continuously to select morphogenic calli on subculture medium containing the same concentration of 2,4-D.

DNA Preparation and Particle Bombardment

Plasmid pBI221 containing β-glucuronidase gene was isolated from *E. coli* DH5α strain. The plasmid map was represented in Fig. 2. In this construct, β-glucuronidase(GUS) gene is regulated by CaMV-35S promoter and nopaline synthase terminator. Plasmid DNAs were digested and electrophoresed to determine the purity. Fig. 3 showed clear banding patterns on 1.5% agarose gel. It was represented that the plasmid map was constructed with the digestion of *Eco*RI and *Hind*III restriction enzymes(Fig. 3). After the preparation of DNA, bombardments were conducted at the presence of 1,100 PSI rupture disk with immature ovules and micro calli.

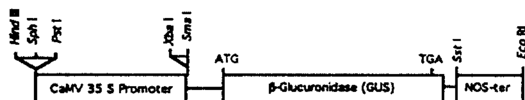

Fig. 2. Diagramatic map of pBI221, the plasmid used in these transformation experiments. The plasmid contains β-glucuronidase (GUS) gene expression cassette consisting of the CaMV 35S promoter and of the nopaline synthase terminator(Nos-ter). The fragment was cloned into the corresponding sites of pUC19.

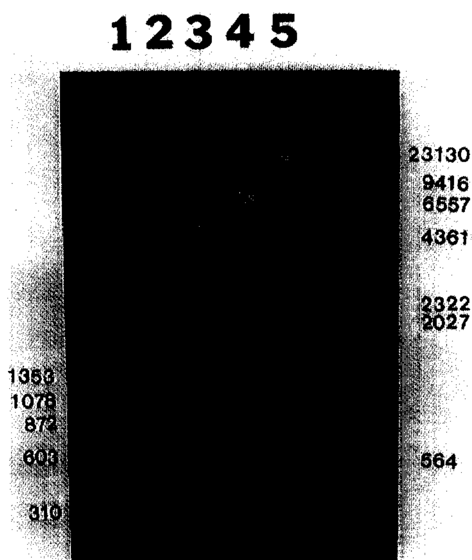

Fig. 3. Restriction map of pBI221 from the culture of E. coli strain(DH5α), the plasmid used in these transformation experiments. The molecular weight markers were φ X174/*Hae*III and lambda *Hind*III on lane 1 and lane 5, respectively. Lane 2 digested with *Hind*III/*Eco*RI represents two different bands ; 3.0kb(35S/GUS) and 2.7kb(pUC19 vector region). Lane 3 shows a linearized plasmid DNA cut with *Eco*RI. Lane 4 is a circular DNA that is uncut.

Bombardment system was demonstrated schematically for the samples(Fig. 4).

GUS Gene Expression

GUS expression was examined after the reactions of bombarded samples and X-gluc. Microprojectile bombardment of both samples with DNA particles resulted in transient expression of GUS gene(Fig. 5). GUS expression was tested with a serial incubation of the substrate and detected as early as six hours of incubation. Immature ovules and micro calli bombarded without plasmid DNA did not show any GUS activities whereas those bombarded with the mixture of plasmid DNA and tungsten particles were revealed GUS gene activity(Fig. 5). The number of GUS expression per specimen was observed under the stereo-microscope. The number of spots

was counted after incubation for 24, 48, and 72 hours in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid(X-gluc). Twenty five(56.8%) among 44 immature ovules bombarded, revealed more than 1 spot and 41(75.9%) of 54 callus samples shown transient expression after all specimen were incubated in X-gluc for 24 to 72 hours at 37℃. Forty-eight hour exposure seemed to be optimal in detecting GUS gene expression. Among the serial bombardments(1 to 3 shots), the expression of GUS gene was significantly different ranging from 1 to 25.75±2.77 for immature ovules of *Populus deltoides*(Fig. 6). Micro calli also produced GUS gene expression upto 11.43±1.22 spots per explant(Fig. 7). As an optimal condition for transient expression and detection, the best combination seemed to be two consecutive bombardments and 48 hour incubation period in X-gluc. In monocot crop plants, similar studies were conducted with intact maize cells to transfer plasmid DNA coding for a selectable marker(neomycin phosphotransferase [NPT II]) and a reporter(β-glucuronidase [GUS]) gene(Klein et al., 1989).

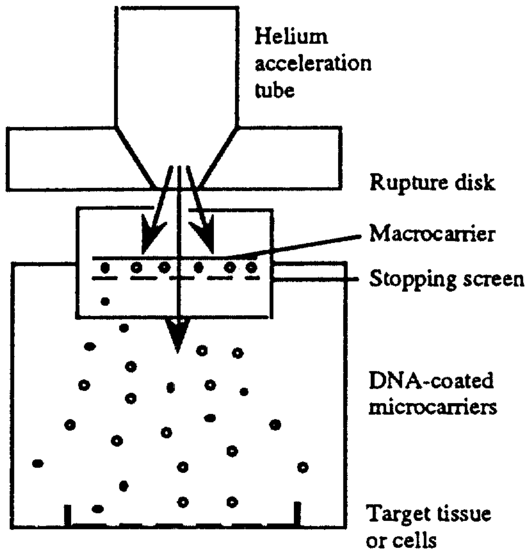

Fig. 4. Schematic diagram of the Helium deriven Biolistics Particle Delivery System(PDS 1000) during activation.

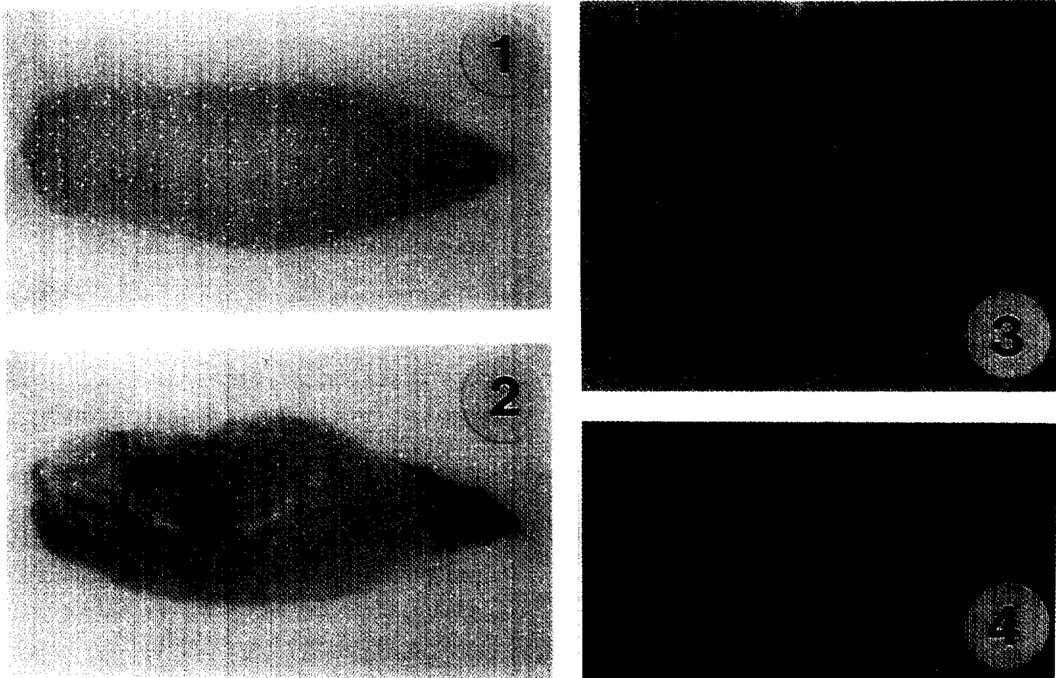

Fig. 5. Histochemical detection of GUS enzyme activity in *Populus deltoides*.
(1)A control of immature ovule, (2)GUS gene expression in immature ovule, (3-4)GUS gene expressed on callus materials.

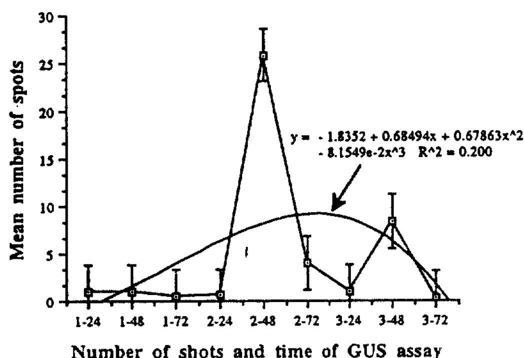

Fig. 6. Effects of the number of bombardments on the expression of GUS gene and the exposure period to X-gluc on the detection of GUS activity. Values are mean number of blue spots after bombardment with the explants of immature ovules. The vertical bars on the graph show the standard error.

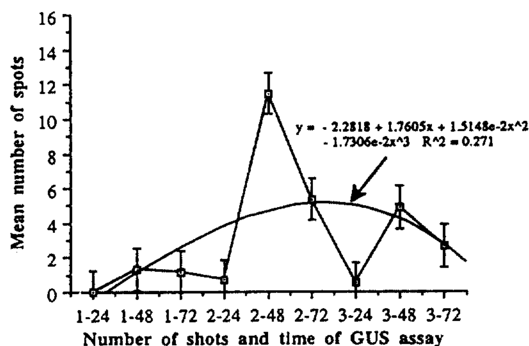

Fig. 7. Effects of the number of bombardments on the detection of GUS gene activity and the exposure period to X-gluc on the detection of GUS gene. Values are mean number of blue spots after bombardment with the explants of calli. The vertical bars on the graph show the standard error.

To determine the important parameters, cotyledon cells of *Pinus taeda* were used to transfer GUS gene by microprojectile bombardment(Stomp et al., 1991). They mentioned that cellular damage sustained by GUS-positive cells ranged from undetectable to sufficiently extensive to cause cell death. The other group transferred the plasmid coding GUS and NPT II into calli and regenerated the whole plants in Yellow poplar(*Liriodendron tulipifera* L. ; Wilde et al., 1992). Two research groups reported gene transformation system as the direct transfer with immature embryos of barley and spring wheat of monocots in angiosperm(Chibbar et al., 1991 ; Kartha et al., 1989). Even if they used different explants as bombardment targets, the results were similar to our studies for the establishment of particle bombardment and the mechanisms to deliver the plasmid DNA for GUS gene expression.

In conclusion, a direct gene transformation system was developed for immature ovules and micro calli of cottonwood species(*Populus deltoides*). To our knowledge, this paper is the first report on the direct DNA delivery via microprojectile-mediated gene transformation and expression with immature ovules and micro calli of pure cottonwood. For successful gene delivery into plant cells and detection after bombardment, the important parameters were (1)the developmental stages of ovules and micro calli, (2)the number of bombardments into targets, (3)the period of incubation with the mixture of target samples and X-gluc. In our results, we addressed the optimal conditions for microprojectile bombardment with the samples of immature ovules and micro calli of cottonwood(*Populus deltoides*).

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