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# Transformation of *Pisum sativum* L. var sparkle; A Non Tissue Culture Method

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## *Agrobacterium tumefaciens*를 이용한 완두(*Pisum sativum* L.)의 형질전환

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## Abstracts

The transfer of genetic material into pea tissue was accomplished by using an avirulent strain of *Agrobacterium tumefaciens* containing the binary vector. The method used for transformation requires non-tissue culture steps as it involves the inoculation of the site of the shoot removed of germinating seeds. The identification of $\beta$-glucuronidase activity in the tissues of $T_0$ pea plants indicates that the plant expressible $\beta$-glucuronidase gene, contained the T-DNA region from pLPBO2, had been transferred at least into somatic tissues. Putative transformed $T_0$ pea plants were advanced to produce $T_1$ plants which were also assayed for the presence of the transferred $\beta$-glucuronidase gene. The presence of the $\beta$-glucuronidase gene in DNAs isolated from $T_1$ plant was demonstrated by DNA gel blot hybridization. This analysis revealed that the transformed plants contained $\beta$-glucuronidase gene.

Key words : Pea, Transformation, *Agrobacterium tumefaciens.*

The abbrebiations used are : GUS, $\beta$-glucuronidase; X-gluc, 5-bromo-4-chloro-3-indolyl-$\beta$-D-glucuronic acid.

## Introduction

For centuries human have been trying to improve quality and yield of their cultivated plants by conventional breeding programs. The transfer of genetic factors from one individual to another has conventionally achieved through sexual breeding, but traditional crossbreeding has the limitation of sexual incompatibility. Gene transfer into plants has opened new ways for the use of recombinant DNA techniques in physiological and molecular studies and may be useful in the future in complementing the conventional breeding programs (Johanna *et al.*, 1989). With a dis-

covery of restriction enzymes and the advent of molecular cloning techniques, today's molecular biologists and plant breeders have available powerful new tools with the potential to change the content or expression of genetic information in plant by transformation. The transfer of genetic material into plant tissues has been attempted by several methods; direct chemically induced DNA transfer (Crossway *et al.*, 1986), electroporation (Fromm *et al.*, 1986), microprojectiles (Klein *et al.*, 1987), or by the more widely used *Agrobacterium* mediated T-DNA transfers (Fraley *et al.*, 1983; Murai *et al.*, 1983).

The development of a method to obtain transformed plants which is independent of the problems induced from tissue culture procedures has been the goal of many laboritories. The *Agrobacterium* mediated transformation is dependent on a large tissue culture effort, and the transformation and regeneration of transformed pea plant may be very difficult. These limitions presently restrict genetic engineering of pea. In this paper, we describe an alternative method for obtaining transformed pea tissue which is based on *Agrobacterium* mediated gene transfer, but it does not require the use of any tissue culture steps.

## Materials and Methods

### Plant materials

The plants used in this study were pea (*Pisum sativum* L.) var sparkle. Seeds were purchased from Jung-ang Seed Co. The seeds were sterilized with 1.5% sodium hypochloride for 20minutes, rinsed three times with sterile distilled water and germinated on plate.

### Bacterial strains and plasmids

*Agrobacterium* strain used in this study was GV3101(pMP90RK)(Koncz and Shell 1986). *E. coli* host strains used in this study were S17.1 and MM294. Plasmids were RK2013(Figurski *et al.*, 1979), pPCV002(Koncz and Schell, 1986) and pBI221. *Agrobacterium* host GV3101 (pMP90RK) was described previously(Koncz and Schell 1986).

### Enzymes and Chemicals

All restriction enzymes and other enzymes used in this study were purchased from KOSCO, Promega, and Phamacia. The [$\alpha$-$^{32}$P] ATP was purchased from New England Nuclear Res. Products. Sodium dodecyl sulfate(SDS), ethidium bromide, lysozyme, agarose, proteinase K, and antibiotics were purchased from Sigma chemical Co. Peptone, yeast extract, agar, and tryptone were purchased from Fluka. The other chemicals used were the first grade and extrapure reagents.

### Bacterial culture and maintenance

*Agrobacterium tumefaciens* strains were grown on YEP(1% yeast extract, 0.5% NaCl, 1% peptone, pH 7.0) at 28℃. *E. coli* strains were grown on LB(0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.5) at 37℃. Bacteria was maintained for periods of a few weeks on the surface of agar media. The plates were tightly wrapped in parafilm and stored at 4℃.

### Plant transformation

Seeds of pea(*Pisum sativum* L.) var Sparkle were sterilized in 1.5% sodium hypochloride solution for 20 minutes, then rinsed three times with sterile distilled water and germinated in sterile petridish lined with moist Whatman filter paper for three days in the dark. Shoot tips were removed and inoculated with *Agrobacterium* strain GV3101 containing the binary vector pLPB02.

### $\beta$-glucuronidase (GUS) activity analysis

GUS enzyme activity assay was performed as described by Jefferson *et al.*,(1987). Tissue ex-

tracts were made from leaf of transformed plants. These were incubated with reaction buffer containing 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) for 24 hrs in a 96 well plate at 37℃.

Genomic DNA Isolation

Plant DNA isolation was performed as previously described (Van *et al.*, 1974). Seedlings after a week, which had been stored in −70℃ defreezer, were ground to a fine powder in prechilled mortar and pestle. This powder was suspended in 10 volumes isolation beffer[20 mM Tris(pH 7.8), 250 mM sucrose, 5 mM $MgCl_2$, 5 mM KCl, 40% glycerol, 0.25% triton X-100, 0.25% β-mercaptoethanol] of that of sample powder. After spining by 10,000xg for 15 minutes at 4℃. The chromatin pellet was then suspended in chromatin suspention buffer (isolation buffer without glycerol), and then extract twice with phenol/chloroform/isoamyl alcohol (50:48:2). The supernatant was collected carefully and add 2 volumes of 100% cold ethanol. DNA was precipitated by centrifugation and washed with 70% ethanol. The pellet was dissolved in 1 ml of TE (pH 8.0).

DNA Gel blot analysis

DNA was isolated from putative transformed leaf issues and from untransformed control leaf tissues described by Van *et al.*,(1974). DNA was digested with *Eco*RI and *Hind* Ⅲ, run on 0.7% agarose gel, and blotted onto nylon membranes (ICN) according to the manufacturer instructions. The filters were hybridized with [$^{32}$P]ATP labeled 2 kb of *Sst* I and *Bam*HI GUS coding region.

DNA Dot Blot hybridization

DNAs were boiled in a total volume of 0.5 ml with a final concentration equal to 0.4 M NaOH, 10 mM EDTA for 10 min. at 95℃. The Zeta-Probe membrane was wet by immersing it in distilled water and assemble the apparatus. DNA samples were put into each well without vacuum.

All wells were rinsed by placing 0.5 ml of 0.4 M NaOH in each then apply vacuum until all wells are quite dry. The membrane was rinsed briefly in 2 X SSC. Prehybridization was done for 5 minutes at 65℃. Hybridization was done for 16 hrs at 65℃ with agitation.

## Results and Discussion

Recombinant plasmid construction

GUS gene from pBI221 was inserted into binary vector pPCV002. The 3 kb GUS fragment was excised with EcoRI and *Hind*Ⅲ and this fragment was inserted into pPCV002 to produce pLPB02, containing multi-cloning site. The plasmid pLPB02 was transferred to *E. coli* and screened on LB plate containing amphicillin.

Introduction of plasmid into *Agrobacterium* via triparental mating.

The binary vector pLPB02 was transferred from *E. coli* to *A. tumefsaciens* GV3101. Binary vector was transferred to *A. tumefaciens* by triparental mating with pRK2013. *Agrobacterium* strain containing an intact binary vector was verified by *Agrobacterium* plasmid quick screening and was used for transformation of pea tissues.

Plant transformation and revelopmemt

Seeds of pea were sterilized in 1.5% sodium hypochloride for 20 minutes, then rinsed three times in sterilized distilled water, and then placed in sterilized petridish lined with sterile Whatman filter paper. After three days, shoot tips were removed and inoculated with *Agrobacterium* using a 1ml syringe. The inoculated explants were

placed on petridish. After a week, shoots were developed at the site of inoculation(Fig. 1). These were transferred to soil for full development.

### Identification of transformed $T_0$ plants

Identification of surviving pea plant which were putatively transformed was accomplished by assaying each plant for GUS activity.

From total 50 plants tested, some plants showed GUS activity (Fig. 3). White arrows indicate GUS positive activity. Lane F was non transformed plants. Thus approximately 10% of the surviving $T_0$ plant showed GUS enzyme activity, suggesting that at least the GUS gene region of the vector was present in the tissue of these pea plant. In contrast, the negative control (lane F) showed no GUS enzyme activity in the leaf of pea.

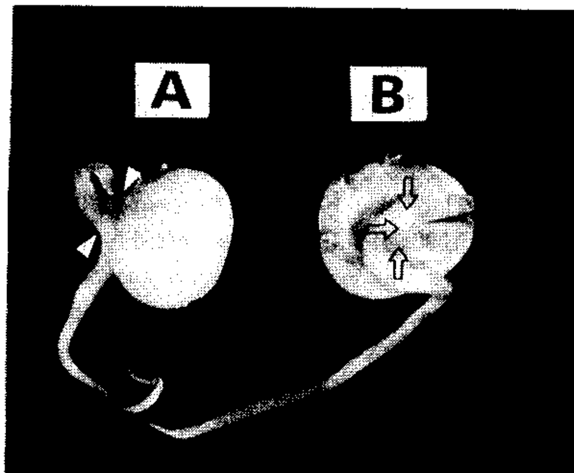


Fig. 1. The inoculation target area of germinated pea seedling. A: three-days-old seedling. White arrows indicate the region where shoot tip was cut. B: Shoots removed seedling. The arrows indicate to the region of bacterial inoculation.

Fig. 2. Shoot regeneration at the area of *Agrobacterium* inoculation. After one week, shoots were regenerated at the area of inoculation.

In addition, no residual *Agrobacteria* could be recovered from leaf segments obtained from the GUS positive $T_0$ plants. This result suggests that the GUS enzyme activity shown in Fig. 3 is not due to the presense of residual *Agrobacteria* in plants.

Thus the GUS enzyme activity observed in these plants was most likely due to the transfer of the T-DNA region from a viable *Agrobacterium* cell into pea tissues near the injection site (Fig. 1, 2). However, these results do not demonstrate that the GUS gene had been integrated within the genome of pea cells. Even if an integration event had occurred it may have been taken place in somatic cells and thus would not be inherited by the seed progeny (Parrot *et al.*, 1989).

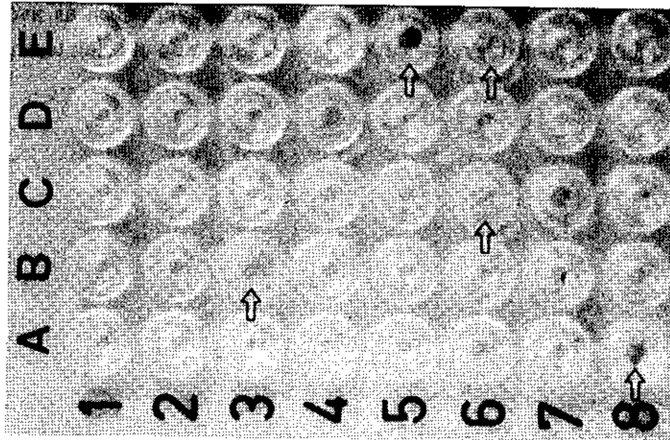


Fig. 3. Gus enzyme activity analysis of $T_0$ pea plants. Arrows indicates GUS positive activity. Lane 1: Untransformed negative control.

### Dot blot hybridization of GUS positive $T_0$ plants

Total genomic DNAs were extracted from leaves of the GUS positive $T_0$ pea plants. The samples were boiled in a total volume of 0.5 ml with a final concentration equal to 0.4M NaOH, 10 mM EDTA at 95℃ for 10min ( see materials and methods). Denatured DNAs were attached to Zeta probe membrane which were hybridized with [$^{32}$P]ATP labelled 2 kb of GUS coding sequence. The positive hybridization results obtained for 12 of the putative transgenic plants have been designated in Figure 4. These results yield information about the molecular composition of the nucleic acids that bind probe material.

### DNA gel blot analysis isolated from $T_1$ Pea plant

Total nucleic acids were extracted from leaves of 3 $T_1$ progeny plants derived from the $T_0$ GUS positive pea plants, and they were digested with *Eco*RI and *Hind* Ⅲ. The positive hybridization results were obtained for 2 of the $T_1$ transgenic plants (Fig. 5). The result that only 2 of the 12 $T_0$ plants yielded transformed $T_1$ plants. The fact that very few of the progeny plants examined have inherited the transferred GUS gene suggests that this gene was either maintained extrachromosomally, or most likely was integrated into the genome of somatic cells of the $T_0$ plant. That is 10 of 12 putative transformed $T_0$ plants contained transformed plant cells which did not yield transformed ovules or pollen cells, thus the transformed phenotype in these plants was because it could not be transmitted to their

progeny.

The development of efficient methods for transforming seedlings would be extremely advantageous because transformed plants could be obtained rapidly without potent of genetic alterations imposed by tissue culture steps. In addition, the most limiting factor for using genetic engineering to complement the conventional pea breeding programs are difficult in regenerating plants in vitro. The results presented here indicate that transgenic pea plants were obtained by *Agrobacterium* mediated transformation of cells of the plumule, cotyledonary node and adjacent cotyledonary tissues of germinating seeds.

The frequency of transformation was about 2 % for obtaining transformed $T_1$ progeny. However, despite the low efficiency of this method for obtaining transformed pea plants, it offers certain advantages such as avoiding tissue culture techniques and in being technically simple.

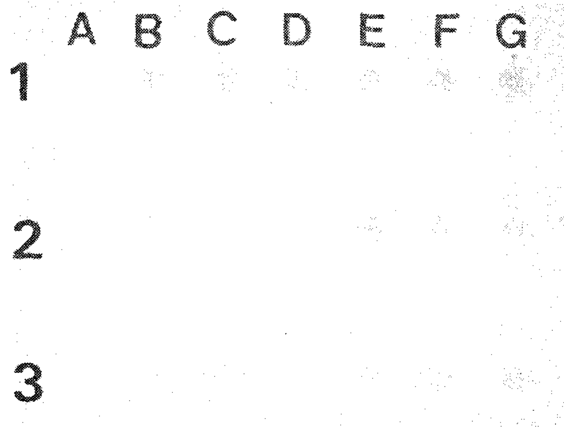


Fig. 4. Dot blot hybridization of GUS positive $T_0$ pea plants. lane A: Negative control. Lane B-G : GUS positive $T_0$ pea plants. Probe was [$\alpha$-$^{32}$P]ATP labeled GUS sequence.

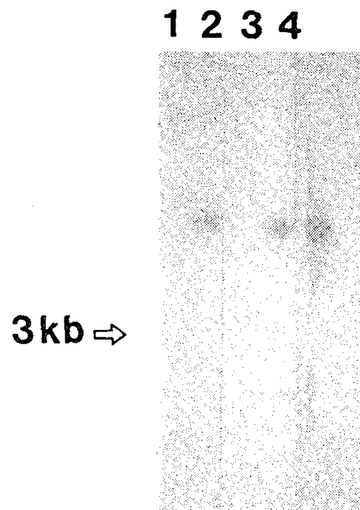


Fig. 5. DNA gel blot analysis of transformed $T_1$ pea plants. Plant DNA was digested with *EcoR* I and *Hind* Ⅲ. Porbe: [$\alpha$-$^{32}$P]ATP labeled 2 kb of GUS coding sequence. Lane 1:3kb of GUS gene fragment. Lane 2, 3: Transformed $T_1$ pea plants.

## 적 요

완두에 있어서 보다 효율적인 형질전환 방법을 모색하고 형질전환된 개체를 얻고자 본 실험을 수행하여 얻어진 결과는 다음과 같다. 형질전환은 발아중인 완두의 생장점(shoot tip)을 제거한 다음 T-DNA내에 GUS gene과 neomycin phosphotransferase Ⅱ gene이 들어있는 binary vector를 가진 *Agrobacterium tumefaciens*를 생장점을 제거한 부위에 감염시켰다. 감염 후 새로 형성된 shoot는 개체당 4~5개 였으며, 그중 GUS유전자가 발현하는 shoot만을 정상적인 식물체로 분화 시켰다. 감염부위에서 형성된 shoot에서의 GUS유전자의 발현빈도는 10%내외였다. 이들 개체로 부터 genomic DNA를 분리하여 Dot blot hybridization분석 결과 T-DNA가 식물체 내에 존재함을 알수 있었고, 수확한 종자를 발아시켜 Sorthern blot hybridization한 결과 T-DNA가 다음세대로 전달되었음이 확인 되었으며 형질전환율은 2% 이내였다.

## References

1. Birnboim, H. C. and J. Doly. 1987, A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7:1513.
2. Crossway, A., J. V. Oakes, J. M. Irvine, B. Ward, V. C. Knauf and C. K. Shewmark. 1986, Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. Mol. Gen. Genet. 202: 179-185.
3. Davis, R. W., M. Thomas, J. Cameron, T. P. John, S. Scherer, and R. A. Padgett. 1980, Methods in Enzymology. Academic Press California pp. 404-411.
4. Fraley, R. T., S. G. Rogers, R. B. Horsch, P. R. Sanders, J. C. Flick. 1983, Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80:4803-4807.
5. Fromm, M. E., L. P. Taulor, and V. Walbot. 1986, Stable transformation of maize after gene transfer by electroporation. Nature 319:791-793.
6. Jefferson, R. A., T. A. Kavanagh, and M. W. Bevan. 1987, GUS fusions: 2-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.
7. Klein, T. M., E. Wolf, R. Wu, and J. C. Sanford. 1987, High-velocity micorprojectiles for delivering nucleic acids into living cells. Nature 327:70-73.
8. Koncz, C., and J. Schell. 1986, The promoter of T-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. 204:383-396.
9. Lehminger-Mertens R. and J. Hans-Jorg. 1989, Plant regeneration from pea protoplasts via somatic embryogenesis. Plant Cell Reports 8:379-382.
10. Maniatis, T., E. F. Fritsch and J. Sambrook. 1982, in Molecular Cloning; A Laboratory Manual, Cold Spring Habor Laboratory, New York.
11. Murai, N., D. W. Sutton, M. G. Murray, J. L. Slightom, D. J. Merlo, N. A. Reichert, C. Sengupta-Gopalah, C. A. Stock, R. F. Barker, J. D. Kemp. and T. C. Hall. 1983, Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222:476-482.
12. Parrot, W. A., L. M. Hoffman, D. F. Hildebrand, E. F. Williams, and G. B. Collins. 1989, Recovery of primary transformants of soybean. Plant Cell Reports 7:615-617.
13. Puonti-Kaerlas, J., P. Stabel, and T. Eriksson. 1989, Transformation of pea (Pisum sativum L.) by *Agrobacterium tumefaciens*. Plant Cell Reports 8:321-324.
14. Van, L. N., G. Engler, M. Holsters, D. E. S. Van, J. Zaenen, R. A. Schilperoot, and J. Schell. 1974, Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature 252:169-170.