Gene Transformation of *Ailanthus altissima* Swingle by *Agrobacterium tumefaciens*

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外來遺傳子에 依한 가중나무의 形質轉換

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SUMMARY

An efficient transformation system was established for *Ailanthus altissima* utilizing the binary system of *A. tumefaciens* strain LBA4404. Callus was initiated from small portions of cambium tissue of *A. altissima* in vitro. Optimum regeneration was achieved with Murashige and Skoog(MS) medium containing 0.01mg/ℓ 2, 4-D, 0.5mg/ℓ BAP, 3 %(w/v) sucrose and 0.75% agar. The multiplication of explants remarkably showed up on medium containing 1.0mg/ℓ BAP. Leaf discs or internodal stem segments were inoculated with *A. tumefaciens* strain LBA 4404 containing the binary vector pPMB 101, which has both β-glucuronidase (GUS) marker gene and neomycin phosphotransferase Ⅱ (NPT Ⅱ) gene. Shoots had been regenerated from 24 lines out of inoculative 50 lines. Transformants were selected by their ability to grow on medium containing kanamycin sulphate (100mg/ℓ). Putative transformation was confirmed by GUS assays. Five GUS -positive plantlets were obtained which confirmed that this marker gene has been transferred into *A. altissima*.

Key Words : *Ailanthus Altissima*, gene transformation, stem segment, *Agrobacterium tumefaciens*, GUS, vector.

INTRODUCTION

Several techniques to introduce naked DNA into plant cell have been described, these include; electroporation (Lindsey et al., 1989), PEG mediated gene transfer(Maas and Wolfgang, 1989), direct gene transfer into protoplasts(Moyne et al., 1989), *Agrobacterium* vec-

tor(Mackay et al., 1988;Charest et al., 1988; Charest et al., 1989;Holbrook et al., 1986).

Agrobaterium tumefaciens is a soil-borne bacterial pathogen which causes crown-gall disease in many dicotyledonous species(De Cleene and De Lay, 1986). During the infection process, a part (T-DNA) of the large bacterial plasmid(Ti -plasmid) is transferred from the bacterium into the host's nuclear genome where it is transcribed. The expression of the T-DNA in the plant cell results in the growth of crown-gall tumours, which continue to grow in the absence of exogenous hormones in culture, and in the production of the characteristic amino acids, the opines (Webb, 1986).

Agrobacterium-mediated transformation has been broadly applicable methods for introduction of foreign DNA into plants and has been successfully applied to woody plants (Fillatti et al., 1987;Mackay et al., 1988; Parsons et al., 1986; McGranahan et al., 1990; Ellis et al., 1989). The host range of *Agrobacterium tumefaciens* was thought to be largely confined to dicotyledonous angiosperms. Until recently there were a few reports of demonstrating transformation by *Agrobacterium* in forest trees.

Agrobacterium tumefaciens-mediated gene transformation in vitro has been reported in a number of tree species such as poplar (Parsons et al., 1986); *Alnus* and *Betula* (Mackay et al., 1988); walnut (McGranaham et al., 1990).

The β-glucuronidase(GUS) gene of *E. coli* has been developed as a gene fusion marker for higher plants by Jefferson et al. (1986). It has been widely used as a reporter gene in the study of foreign gene expression (Hu et al., 1990).

We used a bacterial gene encoding the enzyme β-glucuronidase(GUS) (Jefferson, 1987) in addition to the gene encoding resistance to the antibiotic kanamycin in a binary vector system of *Agrobacterium* strain LBA4404. It serves as a marker of successful transformation because it induces change in the transformants that can be assessed quickly and simply (Graham et al., 1990).

Regenerants or plantlets from callus in woody plants were not facilitated (Vahala et al., 1989). Regenerants provided from organogenic calli which has been made more efficient by using a more virulent strain of *Agrobacterium* and vectors containing genes for both kanamycin resistance and β-glucuronidase(GUS) activity.

Ailanthus altissima is a particulary interesting plant species because of its value as an worldwide shade tree, fast growth on the barron soil, insect resistance, tolerance of air pollution, useful avenue and also it has alkaloids as ketoglutarate (Aragozzine et al., 1988). Little progress, however, has been made in the development of techniques for the transformation of the *A. altissima*.

The objectives of this work were to determine whether GUS gene products were expressed or not and then reliably detected or not in transformed *Ailanthus altissima* tissue when adventitious buds are exposure shortly to kanamycin after inoculation with the *Agrobaterium* in order to enhance the frequency of transgenic shoots.

MATERIALS AND METHODS

Plant materials

The calli of *A. altissima* were initiated on MS basal medium supplemented with various growth regulators. By the use of 2, 4-D(2, 4-dichlorophenoxyacetic acid) and BAP(N^6-benzyl amino purin), the growth of callus was much fast. Friable callus which had been derived from cambium was regenerated as described by Park(1989). Shoots derived from callus were elongated on MS basal medium supplemented with 3%(weight/volume) sucrose, 0.7% agar, pH 5.8 without growth regulators.

Continuously, internodal stem segments for multiplication were placed on MS basal medium with 3%(W/V) sucrose, various concentration of BAP (0.0, 0.02, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.

0, 20.0 mg/ℓ separately) during 30 days. One to three months after establishing the materials, side branches extending from axillary buds were used in the transformation experiments.

Bacteria strain

The avirulent *A. tumefaciens* strain LBA4404 containing the binary plasmid pPMB101 was used for all transformation experiments. The double cut fragments of β-glucuronidase gene from pBI221 and pPCV002 were ligated with T$_4$ DNA ligase. The construction of pPMB101 is shown in Figure 1.

A. tumefaciens strains were kept on selective YEB plates containing 25mg/ℓ kanamycin and 100mg/ℓ ripampicin.

The stirans were grown in liquid YEB medium with antibiotics diluted 1:10 and subsequently grown during overnight in YEB medium.
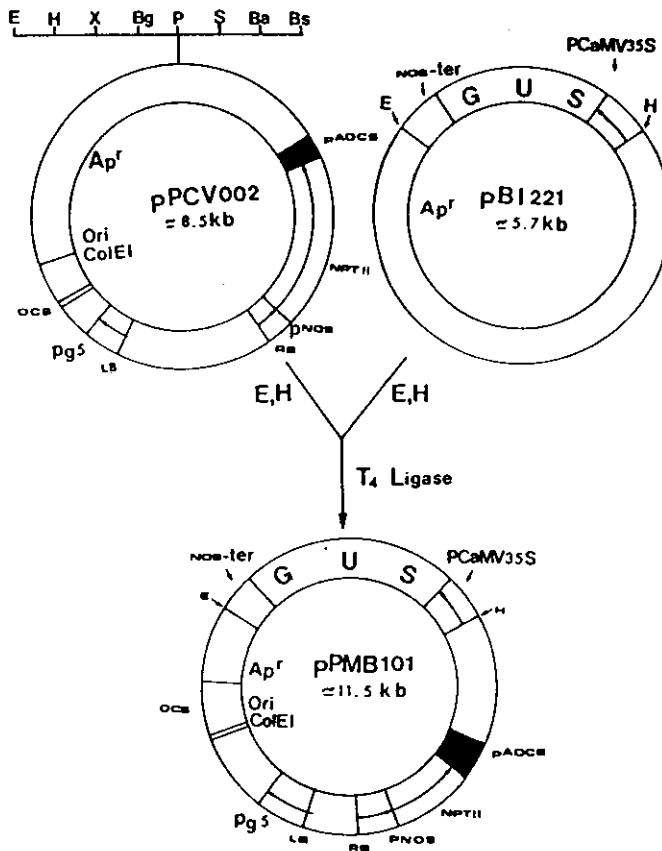

Fig. 1. Construction of pPMB101
The 3kb Hind Ⅲ and Eco R Ⅰ fragment containing β-glucuronidase(GUS) gene of pBI 221 was digested by restriction enzyme Hind Ⅲ and Eco R Ⅰ and ligated to pPCV002 digested with Hind Ⅲ and Eco R Ⅰ. NPT Ⅱ (Km); Kanamycin resistant gene for plant, CaMV 35S promoter; strong plant promoter, NOS poly(A); poly adenylation signal of nos gene, H; Hind Ⅲ, E; Eco R Ⅰ, X; Xba Ⅰ, Bg;Bgl Ⅱ, P;Pst Ⅰ, S;Sal Ⅰ, Ba; Bam H I, Bs; Bst E Ⅱ, RB; Right border, LB; Left border

Inoculation of bacteria

In vitro axillary buds and leaf discs of explant were wounded with scalpel or needle and bacteria were inoculated into the wounds. After infection, they were cultured on regenerating medium at 25 ± 1℃ with an 16 hours in photoperiods and then regenerated shoots were counted.

Kanamycin resistance assay

Regenerated explants were cultured on MS basal medium containing the selection agent kanamycin sulphate ($100mg/\ell$) to identify the transformants. The selected plants were subsequently transferred to shoot elongation medium without growth regulators.

GUS assay

Plant tissue was assay histochemically for β-glucuronidase(GUS) activity according to Jefferson(1987) using an extraction buffer containing 100mM $NaPO_4$, pH 7.0, 10mM Na_2EDTA, 0.025 % thimerosal and 0.1% Triton X-100. To 1mℓ of this buffer 10 $\mu\ell$ and 25 $\mu\ell$ X-Gluc(5-bromo-4-chloro-3-indolyl β-glucuronidase in dimethylform-amide) was added.

Putative transformed plants were tested for expression of the GUS gene by cutting a small piece from leaf surface and immersing it in X-Gluc solution in multi well plates at room temprature or 37℃.

If the piece developed a distinct blue color at leaf section from which it was cut was multiplied on the medium with $0.5mg/\ell$ BAP as a subclone for retesting.

RESULTS

Plant material

The small section of cambium tissue gave rise to callus growth after 3 weeks on solid agar medium supplemented with various growth regulators.

The growth of callus in high level(above $1.0mg/\ell$) of 2, 4-D was faster than that of low level (below $0.5mg/\ell$) of 2, 4-D at concentration. Cytokinin alone was not able to induce callus and the best conditions of callus induction and growth occurred on the MS medium supplemented with 2, 4-D $1.0mg/\ell$ and BAP $0.1mg/\ell$.

To determine the effect of growth regulators on plant regeneration, callus was cultivated on MS medium supplemented with various concentration of 2, 4-D and BAP for 8 weeks(Table 1). Fragments of these calli changed into green color when subcultured on medium containing 2, 4-D and BAP. Healthy green shoot buds emerged from the callus. Although this organogenic callus was transferred to media with BAP as the sole growth regulators, the number of shoots did not increased rather than that of 2, 4-D and BAP. The most numerouse shoots was observed at $0.01mg/\ell$ 2, 4-D and $0.5mg/\ell$ BAP.

Table 1. The number of shoots derived from callus cultured on MS medium with different concentrations of 2, 4-D and BAP for 8 weeks.

Growth regulators(mg/ℓ)		Number of shoots (Mean±SE)
2, 4-D	BAP	
	0.10	2.0±0.58*
	0.25	2.7±0.89
0.00	0.50	3.4±1.45
	0.75	2.3±0.33
	1.00	2.3±1.45
	0.10	—**
	0.25	2.7±0.67
0.01	0.50	5.0±1.15
	0.75	4.0±0.58
	1.00	2.0±0.34

*   Mean ± SE of 3 replications
* *   No response

Addition of BAP to the MS medium in the range of 0.02–20mg/ℓ resulted in various degree of multiple shoot formation from explants. Maximum multiple shoots were observed in the medium of 1.0mg/ℓ BAP(Table 2).

Regenerated shoots have a tendency to form callus at the base. For this reason, any of growth regulators were not used in rooting medium and small roots were formed within several weeks.

Table 2. Effects of different concentrations of BAP on shoot multiplication from cuttings of *Ailanthus altissima* after 4 weeks in culture.

Conc. of BAP(μM)	0.0	0.02	0.1	0.2	0.5	1.0	2.0	5.0	10.0	20.0
No. of shoots	–	2.5	2.8	5.0	5.3	10.0	6.0	5.5	3.0	0.5

Plant transformation

Attractive results were obtained by application of bacterial cultures, which made use of wounded parts produced by needles on adventitious bud. But leaf discs which were not infected with *A. tumefaciens* failed to regenerate of medium with cytokinin. This result shows that leaf disc is very sensitive to wounds by needle and to kanamycin.

As shown in Table 3, the frequency of shoot regeneration was 49%(Table 3). Development of shoot from axillary bud is shown in Fig. 2A. In this case, shoot regeneration was very rapid (2–3 weeks). It is clear that yield of regeneration obtained by this procedure is much better than that of being obtained by procedure reported previously.

Kanamycin resistance assay

Kanaycin resistance was assayed based on the ability to subsequent growth of regenerated shoots on medium containing 100mg/ℓ kanamycin. Untransformed shoots showed inhibition of shoot growth and bleaching of plant tissue and then died (Fig. 2. B-2). Putative transformants with the binary vector continued to grow on medium containing kanamycin (Fig. 2. B-1).

Most of regenerated and transformed plants developed root also. The regenerated plants thus showed that their form and behaviors were similar to their transformed "mother plant". On the contrary, non transgenic, control plantlets were inhibited in root development on MS medium supplemented with 100mg/ℓ kanamycin (Fig. 2. B-2).

The plants of kanamycin resistant derived from axillary bud were cultured on hormone-free medium and were continuously multiplied on medium containing 1.0mg/ℓ BAP (Fig. 2. C & D).

GUS assay

GUS-positive shoots were easily detected within 4 hours of exposure to the X-Gluc substance. The distinct blue color could be first observed on cut surfaces or areas damaged in handling (Fig 2. E).

Table 3. Frequency of transformation axillary buds of *Ailanthus altissima* cocultivated with *A. tumefaciens*.

No. of shoot inoculated	No. of regenerated shoot	No. of transformed shoot
50	24 (49%)	5 (10%)

As the GUS-positive lines were multiplied, they were retested for GUS activity and exam-

ined for the presence of contaminating bacteria. Shoots have been regenerated from 24 lines out of 50 lines which had been inoculated. Of the 24 lines tested, 5 lines(10%) expressed GUS activity (Table 3).

DISCUSSION

This paper describes protocols for the regeneration and transformation of *A. altissima*. Regeneration protocols are essential for the development of successful transformation systems (Atkinson et al., 1991). Usually most of the regeneration protocols take at least several months.

But transforamtion of shoot using adventitious bud has the advantage that the primary and secondary axillary buds could directly develop into shoots without an intervening callus phase. It will be solved that leaf disc were not in directly contact with selective medium containing knamycin. This results were similar to the research of Schrammeijer et al. (1990). Ishida et al. (1989) reported that infection of lateral parenchyma cells surrounding the bud apex gave good effect.

The binary Ti-plasmid vector, pPMB101 which has both the NTP Ⅱ and β-glucuronidase genes provided a more efficient identification system for the occurrence of transformation (Graham et al., 1990). Phenotypic variation among transformed plants was observed. Such variation may result from differences in location of the insertion site of the DNA within the plant genome rather than from differences in the number of gene copies intergrated into the genome (Thomas et al., 1989; Uematsu et al., 1991).

Plantlets tested in X-Gluc solution after inoculation had abundant bacteria on thier surfaces which interfered with detection of transformed tissues. The clearing of bacteria was not Possible by cefotaxim rinse before plating.

Molecular biological research in more detail will be undertaken for transformants in the future and inheritance of the incorporated genes must be studied.

In this paper, we have demonstrated that *Ailanthus altissima* can be regenerated from axillary buds and efficinetly transformed using *A. tumefaciens*. These results suggest that *A. altissima* has potential for using as a model system of transformation.

摘　要

본 연구에 사용한 시료는 형성층 유래의 캘러스를 0.01mg/ℓ 2, 4-D, 0.5mg/ℓ BAP, 3% sucrose, 0.75% 한천을 첨가한 MS배지에서 재분화 시킨 개체를 이용하였다. 시료의 대량증식은 1.0mg/ℓ BAP를 첨가한 MS 배지에서 실시하였으며 엽전개는 식물생장조절제가 첨가되지 않은 MS배지로 옮겨서 실행하였다. Agrobacteria를 이용한 형질 전환은 엽절편, 절간조직등을 박테리아를 묻힌 침으로 자극하여 식물체 분화를 유도하였다. 그 결과 엽절편 조직에서는 분화된 식물체를 얻지 못했으나, 절간조직의 측아에서는 49%에 달하는 24개체가 분화되었다. 이들 분화된 줄기는 kanamycin이 100mg/ℓ이 함유된 선발 배지에서 일차적인 선발을 하여 최종적으로 GUS 유전자 검정을 한 결과 처음에 접종한 50개체중 형질전환 된 것으로 추정되는 5개체를 얻어서 형질 전환 추정 비율은 10%에 달한 것으로 나타났다.
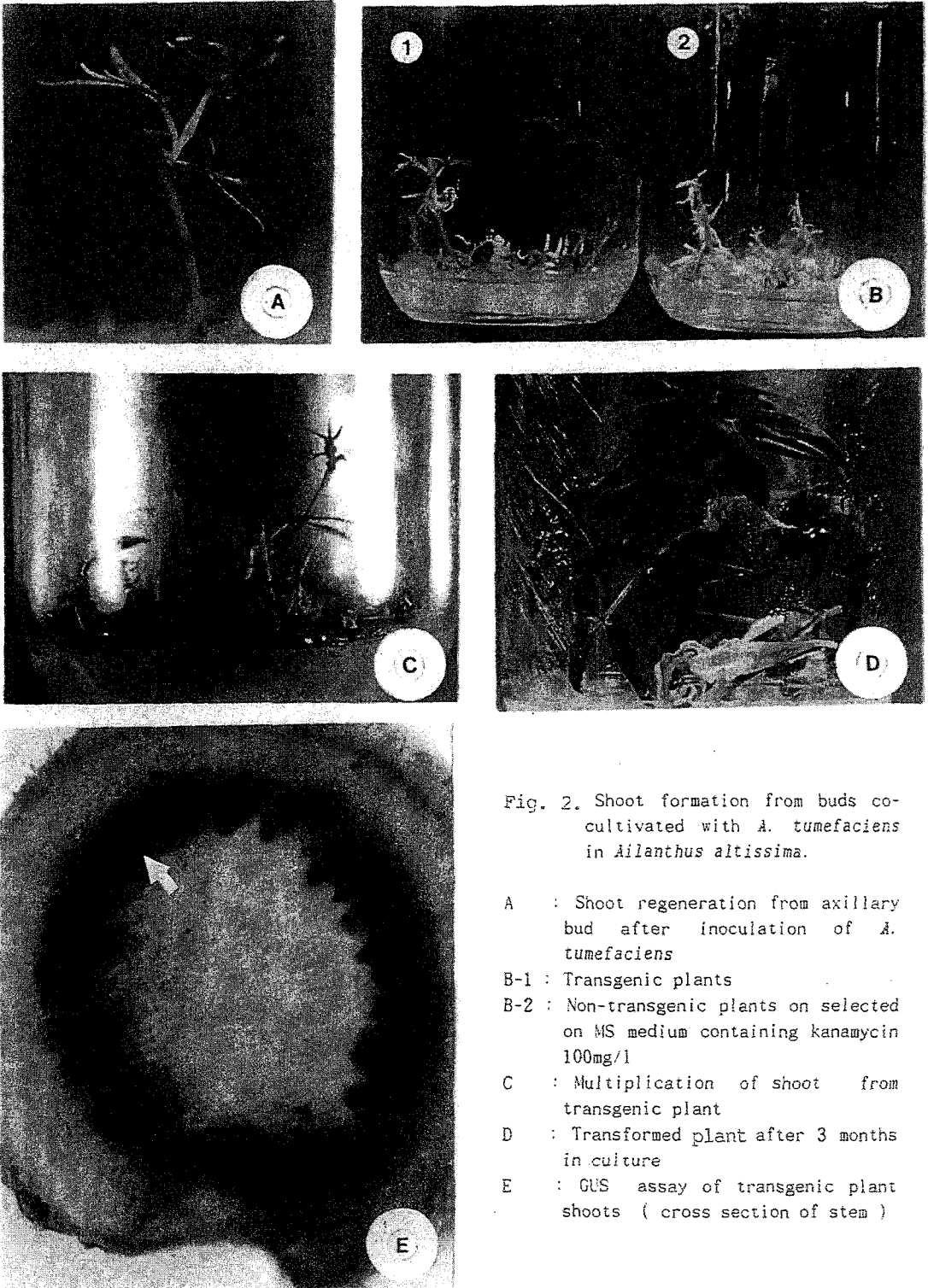
Fig. 2. Shoot formation from buds co-
cultivated with *A. tumefaciens*
in *Ailanthus altissima*.

A   : Shoot regeneration from axillary
bud after inoculation of *A.*
tumefaciens
B-1 : Transgenic plants
B-2 : Non-transgenic plants on selected
on MS medium containing kanamycin
100mg/l
C   : Multiplication of shoot from
transgenic plant
D   : Transformed plant after 3 months
in culture
E   : GUS assay of transgenic plant
shoots (cross section of stem)

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