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# *Agrobacterium tumefaciens*-mediated transformation of soybean (*Glycine max* (L.) Merr.) is promoted by the inclusion of potato suspension culture

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**Abstract.** We have established an effective system for increasing the transformation frequency of soybean mediated by *Agrobacterium*. Soybean cotyledons were excised and infected with *Agrobacterium tumefaciens* strains C58C1 (pGV2260::NG1) and C58C1 (pGV2260::NG3) which contain both of the GUS and NPT II genes. Established potato suspension cells (PSC) was found to be able to increase significantly the transformation efficiency when added in the infection medium. Transformed callus was increased as high as 70% after treated by PSC in this study, the frequency was higher than that treated by acetosyringone (AS). NPT II activity and GUS protein were detected in the transformed callus and plants, thus indicating that the chimeric genes were successfully integrated and expressed in the transgenic tissues. High levels of phenolic compounds such as sinapinic acid and acetosyringone were found in the potato suspension cells. Their roles on promoting the transformation efficiency were also discussed.

**Key words:** Acetosyringone; *Agrobacterium tumefaciens*; *Glycine max*; Phenolic compounds; Potato suspension cultures; Sinapinic acid; Soybean; Transformation.

## Introduction

*Agrobacterium tumefaciens* is a convenient vector for transferring foreign genes into dicotyledonous plants (Klee *et al*., 1987; Hooykaas, 1989). Soybean (*Glycine max* L.) is, however, well known to be less sensitive to *Agrobacterium tumefaciens* infection (Owens and Cress, 1985), although a few cases of transformation mediated by *Agrobacterium tumefaciens* in soybean have been reported (Pedersen *et al*., 1983; Owens and Smigocki, 1988; Delzer *et al*., 1990). Transgenic plants have also been obtained by using the GUS-neo chimeric gene (Hinchee *et al*., 1988; Parrot *et al*., 1989). However, many problems concerning transformation still extist in soybean such as the low transformation efficiency and low rate of regeneration which limit the application of *Agrobacterium*-mediated gene transfer (Byrne *et al*., 1987; Delzer *et al*., 1990).

Virulence induction of *Agrobacterium* was known to be one of the important steps in plant tumor formation (Garfinkel and Nester, 1980; Ooms *et al*., 1980). Hooykaas and Schilperoort (1984) suggested that the activation of virulence genes of *Agrobacterium* is an essential step for T-DNA transformation. They also showed that products of these virulence genes can act in trans to mobilize T-DNA from Ti plasmid to plant genomes (Hooykaas, 1989). Several scientists found that phenolic compounds such as acetosyringone and precursors of lignin (ex. coniferyl alcohol and sinapinic acid) released from wounded plant tissues can act as inducers for the expression of virulence genes (Stachel

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*et al.*, 1985, 1986; Melchers *et al.*, 1989; Spencer and Towers, 1988). Bolton *et al.* (1986) reported that the mixed phenolic compounds could increase more efficiently the expression of virulence genes than the compound used individually. Some of plant metabolites such as glycine betaine were also shown to be able to enhance the AS-mediated induction of virulence genes at low pH (Vernade *et al.*, 1988). These results implied that certain kinds of plant cells can produce some metabolites which may enhance the *Agrobacterium* transformation system. In this study, we demonstrated that the transformation efficiency can be increased by adding potato suspension cells or acetosyringone to the *Agrobacterium* infection medium. In addition, the contents in such potato suspension cells which were possibly related to this virulence induction were also discussed.

## Materials and Methods

### *Plant Materials*

*In vitro* cultured plants of potato (*Solanum tuberosum* L. var. ADH69), tobacco (*Nicotiana tabacum* L. var. Taiwan's native cultivar-TT7), and soybean (*Glycine max* L.) were used in this experiment. Potato and tobacco plants were routinely subcultured in MS medium (Murashige and Skoog, 1962) once per 6 weeks. These cultures were maintained at 25°C and 16 h light under 3000 lux illumination. Seeds of soybean varieties-ADOC, G89, and G98 were germinated on MS medium at 25°C and 16 h light under 100 lux illumination. Two days old seedlings were moved to the light condition with 2000 lux illumination for further growth.

### *Initiation of Potato Suspension Cultures*

Stems of *in vitro* cultured potato plant (var. ADH69) were cut into 1 cm segments and placed on PCS medium (each liter contains MS salts, 1 g casein hydrolysate, 40 mg adenine sulfate, 0.4 mg GA3, 3 mg 2,4-D, 0.3 mg kinetin, 100 mg myo-inositiol, 0.25 mg 6-BAP, 15 mg L- glutamine, 0.5 mg folic acid, and B5 vitamins, pH 5.8) for callus formation. After 2 weeks, approximately 0.1 g of potato calli were transferred to 25 ml liquid PCS medium in 125 ml Erlenmeyer flasks for the growth of suspension cells. The suspension cultures were maintained at 25°C on an orbital shaker (120 rpm) and were subcultured once each month with PCS liquid medium. The suspension cultures were kept for such a long time to obtain enough plant phenolic compounds which could be released from potato suspension cells into the medium. These suspension culture media were used to detect the phenolic compounds by HPLC and acted as infection medium in transformation procedure.

### *The Determination of Phenolic Compounds*

*In vitro*-cultured plants of potato (ADH69), tobacco (TT7) and soybean (ADOC), and the potato suspension cultures were sampled and analyzed for wall-derived phenolic compounds. Each plant tissue was cut into 0.1-0.2 cm segments and put into the 50 ml -flask with 10 ml PCS medium. After 5 days incubation, the solution was filtered through 0.22 $\mu$m nitrocellulose (Corning Laboratory Science Company, USA). The filtered solution was then partitioned with 2.5 ml chloroform, and the chloroform phase was collected. The water phase was partitioned again with another 2.5 ml chloroform. Mix these two chloroform fraction and evaporated to completely dry under vacuum. The residue was then dissolved in 500 $\mu$l methanol. Twenty $\mu$l of methanol solution was injected into High performance liquid chromatograph (HPLC) system (Waters, model 510, USA) at 280 nm for the determination of phenolic compounds.

### *Bacterial Strains*

*Agrobacterium tumefaciens* C58C1 (pGV2260::NG1) and C58C1 (pGV2260::NG3) (kindly provided by Dr. Marc Van Montagu, Laboratorium Voor Genetica, Belgium) were used for all the infections. The bacteria were grown overnight in 5 ml YEB medium (Chang and Chan, 1991) containing 100 $\mu$g/ml rifapicin, 100 $\mu$g/ml carbenicillin, 100 $\mu$g/ml spectinomycin, and 300 $\mu$g/ml streptomycin. The cointegrated Ti plasmid: pGV2260:: NG1 carrying neomycin phosphotransferase II (NPT II), octopine synthase (OCS), $\beta$-glucuronidase (GUS), and hygromycin phosphotransferase (hpt) genes was used as infection vector. pNG3 was constructed by inserting the chimeric GUS, NPT II, and HPT genes from pGV2260.

### *Plant Transformation*

The explants of cotyledon excised from 8-12 days old seedling were wounded by scalpel which had been previously immersed in the medium of *A. tumefaciens*. The wounded tissues were placed on petri dish contain-

ing 10 ml potato suspension cultures (cultured for one month) with 25 $\mu$l *Agrobacterium* cultured in darkness for 3 days. The bacterial concentration was determined by measuring the absorbance at 600 nm with spectrophotometer ($A_{600}$=0.6 to 0.8). Around $8\times10^7$ cells per ml of the bacteria was used for each inoculation. The infected tissues were transferred to modified $B_5BA$ medium (each liter contains $B_5$ salts, $B_5$ vitamins, 0.5 mg 2,4-D, 1 mg 6-BAP, 500 mg cefatoxime, and 100 mg kanamycin, pH 5.6). After 4 weeks growth, the shoots formed from transgenic calli were excised and transferred to $B_5$ medium containing the same concentration of antibiotics. Transgenic plants with regenerated shoots and roots were transferred to sterilized soil for further growth. To compare the transformation efficiency, we added 200 $\mu$M AS to *Agrobacterium* cultured overnight, and then infect the soybean cotyledon with such treated *Agrobacterium*. The continued transformation procedure was proceeded according to the protocol described above.

*NPT II Enzyme Assay*

Leaves or calli of transgenic plants were assayed for the expression of the NPT II gene by dot-blot method according to Radke *et al.* (1988) with some modifications. For the NPT II assay, samples were assayed at least for four replicates. Leaf tissue (50-100 mg • fw) was grounded in 1.5 ml eppendorf tube with equivalent amount (50-100 $\mu$l) of extraction buffer (2.5 mM Tris, pH 6.8, 0.143 mM $\beta$-mercaptoethanol, 0.27 mM leupeptin), and then centrifuged for 15 min at 4°C. Twenty $\mu$l of the supernatant was mixed with 10 $\mu$l of reaction buffer A (67 mM Tris-maleate, 42 mM $MgCl_2$, 400 mM $NH_4Cl$, 1.7 mM dithiothreitol, 0.4 mg/ml kanamycin sulfate) or reaction buffer B (identical to reaction buffer A except for kanamycin). Five $\mu$l of ATP solution (1.0 $\mu$ Ci [$\gamma$-$^{32}$P] ATP (Du Pont company, USA) per 5 $\mu$l, 0.75 mM ATP in buffer B) was added to each reaction mixture. The samples were incubated in 30°C water bath for 30 min. Using a "Hybri-Dot" blotting apparatus (BRL, USA), samples were blotted onto three layers of Whatman P81 ion exchange papers placed on top of one Whatman 3 mm paper. All the papers were washed twice with distilled water for 4 min. After that, the blots were incubated for 60 min at 65°C in 10 ml proteinase K solution (1.0 mg/ml proteinase K in 1% SDS), and washed subsequently once with distilled water for 4 min and then washed three times with 85°C distilled water. After drying, blots were exposed to X-ray film (Kodak, USA) with an intensifying screen.

*GUS Protein Detection*

GUS protein was detected by 7.5% SDS-PAGE according to Jefferson (1988). GUS was extracted from transgenic tissues with GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Trixton X-100, 0.1% sarkosyl, 10 mM $\beta$-mercaptoethanol). Plant extracts (10-50 $\mu$l) with an equal volume of SDS sample buffer (62.5 mM Tris-HCl, 0.23% SDS, 10% glycerol, 50 mM $\beta$-mercaptoethanol, 0.001% bromophenol blue) was incubated at room temperature for 10-15 min. Electrophoresis was run overnight at 50 V. Washed the gel with 100 ml of GUS extraction buffer for four times within 2 h. The gel was then incubated on ice with GUS fluorometric buffer (1 mM methyl umbelliferyl glucuronide in GUS extraction buffer) for 30 min, the gel was incubated at 37°C for 30 min under darkness. The reaction was stopped with 0.2 M $Na_2CO_3$ and the gel was photographed with a Kodak 2E Wratten filter (Kodak, USA) under UV plate.

## Results

*Levels of Phenolic Compounds in Wounded Tissues and Potato Suspension Cultures*

The phenolic compounds released from wounded plant tissues were assayed by high performance liquid chromatography (Table 1). Results showed that the contents of phenolic compounds in potato or tobacco were 10 times more than that of soybean and the amount of these released compounds were different in

**Table 1.** *The content of acetosyringone (AS) in various tissues of potato, tobacco, and soybean (unit: μg/g tissues)*

| Tissues | Potato (cv. ADH69) | Tobacco (cv. TT7) | Soybean (cv. ADOC) |
|---|---|---|---|
| Root | 100.8 | 1007.6 | 142.2 |
| Microtubes | 1615.8 | – | – |
| Stem | – | 1941.2 | 45.8 |
| Leaf | 1473.7 | 814.2 | 152.9[a] |
| Total | 3190.3 | 3763.0 | 340.9 |

[a]Cotyledon.

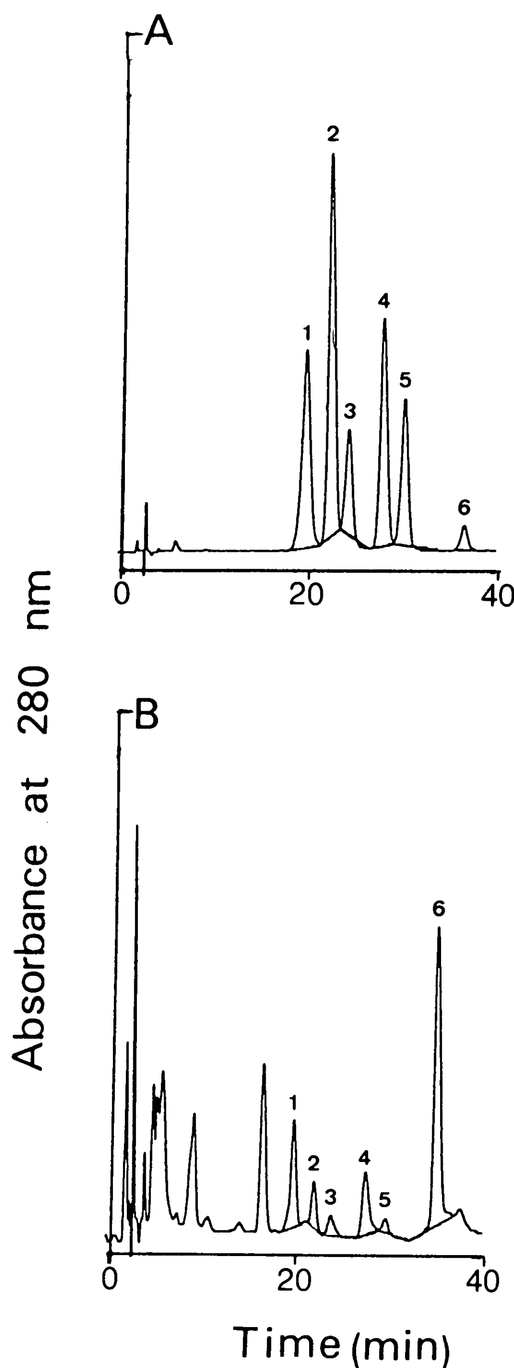


Fig. 1. Chromatographic fraction profile of phenolic compounds assayed from potato suspension culture medium by HPLC. A: standard; B: 20 day-cultured potato suspension medium. Peak 1, syringic acid; Peak 2, 4'-hydroxyacetophenone; Peak 3, syringaladehyde; Peak 4, acetovanillone; Peak 5, acetosyringone; Peak 6, sinapinic acid.

various tissues. In potato, microtubes and leaves released 1615.80 and 1473.70 μg/g•fw of AS, respectively, whereas roots released relatively the low level of AS (100.80 μg/g • fw). High contents of AS were detected from tobacco root and stem tissues. However, as compared with potato and tobacco, soybean tissues produced very few amount of AS (Table 1).

When the potato suspension culture was determined for phenolic compounds (Fig. 1), it was found that the amount of sinapinic acid (SA) was the highest among all the detected phenolic compounds (Table 2). Besides, we also found that these two specific compounds (SA and AS) were accumulated to the highest level on the 30th day and 60th day, respectively, following subcultured and then decreased thereafter.

**Table 2.** *The content of phenolic compounds in potato suspension cultures (μg/ml)*

| Phenolic compounds | Culturing time (DAYS) | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 30 | 60 | 90 | 120 | 150 |
| SYA | 9.5 | 1.2 | 0.9 | 7.6 | 63.3 | 1.9 |
| SAD | 3.0 | 2.1 | 0.2 | 3.4 | 73.0 | 0.9 |
| HAP | 4.2 | 3.6 | 0.0 | 9.2 | 118.8 | 30.6 |
| AV | 50.1 | 35.8 | 0.7 | 10.2 | 17.1 | 39.4 |
| AS | 14.3 | 54.8 | 1864.3 | 5.1 | 7.6 | 3.2 |
| SA | 1011.3 | 1526.9 | 735.6 | 99.7 | 43.3 | 15.9 |

SYA=syringic acid; SAD=syringaladehyde; AV=acetovanillone; HAP = 4-hydroxyacetophenone; AS = acetosyringone; SA = sinapinic acid.

*Enhancing Effects of Potato Suspension Cultures and AS on Transformation Efficiency*

Soybean cotyledon was excised and infected as described in Materials and Methods. The addition of potato suspension culture to the infection medium significantly increased the forming frequency of transformed calli up to 64% (32/50), whereas the controls had only 3.3% (1/30) (Table 3). The addition of 200 μM AS to the bacteria solution also increased the transformation frequency to 35.5% (22/62), and the control was only 3.3% (1/30) (Table 3). Following the coculture with bacteria, the infected cotyledon were transferred to the selection medium. Transformed calli were observed and the wounded site of infected cotyledon after inoculated for one week, whereas non-transformed explants could not be alive following the continuous selection by kanamycin. Only a few of transformed calli were regenerated into transgenic plants. The green

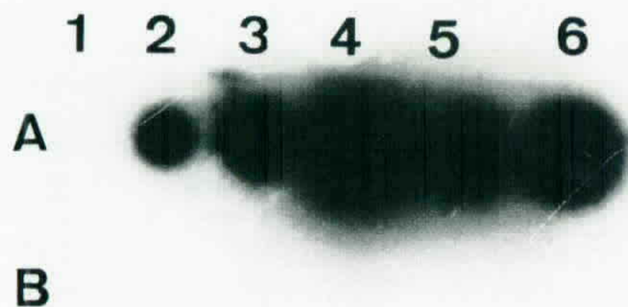


Fig. 2. Detection of the NPT II activity by Dot Blot assay. Row A: the reactions containing kanamycin. Row B: the reactions without kanamycin. Lane 1, leaf tissue from untransformed plant (control); Lane 2, the activity of NPT II enzyme in leaf extracts of transgenic plant; Lane 3-6, the expression of NPT II enzyme of transgenic callus.

**Table 3.** *Transformation frequency of soybean mediated by* ***Agrobacterium tumefaciens*** *C58C1 (pGV 2260::NG1)*

| Treatment | Varieties | Cot[a] | Calli | Plant[b] |
|---|---|---|---|---|
| PSC[c] | ADOC | 50 | 32 | 3 |
| | G89 | 25 | 18 | 0 |
| | G98 | 25 | 16 | 0 |
| AS[d] | ADOC | 62 | 22 | 0 |
| | G89 | 25 | 8 | 0 |
| | G98 | 25 | 7 | 0 |
| Control | ADOC | 30 | 1 | 0 |
| | G89 | 30 | 2 | 0 |
| | G98 | 30 | 1 | 0 |

[a] Number of soybean cotyledon infected.
[b] Number of transgenic plant.
[c] PSC=inoculation with potato suspension cultures.
[d] *Agrobacterium* cultureds overnight with 200 $\mu$M AS and added 25 $\mu$l to 10 ml PCS infection medium.

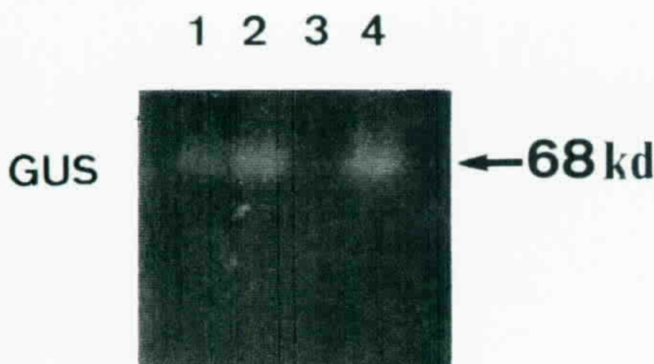


Fig. 3. Detection of GUS by SDS-PAGE. Lane 1, extract from transgenic callus; Lane 2, extract from transgenic plant; Lane 3, non-transformed soybean plant; Lane 4, standard from *E. coli* (800 unit). Twenty $\mu$g protein were loaded in lane 1-3.

calli and leaf tissues of transgenic plant could also express positively the NPT II activity, whereas no response of NPT II was detected in controls (Fig. 2). Although no GUS activities were detected in non-transformed tissues, some older tissues such as roots and wounded tissues were found to accumulate some fluorigenic compounds which might be produced by contamination. These compounds can be removed by adding polyvinyl pyrollidone in the GUS extraction buffer, which was then eluted by a brief spin column of sephadex G-25. GUS expression in the transgenic plants or transgenic calli were confirmed again for gene transfer by *in situ* localization with SDS-PAGE (Fig. 3). These assays confirmed the fact that the chimeric genes had been integrated and expressed in the transgenic tissues.

## Discussion

The activation of virulence genes is an essential step for the T-DNA transfer from Ti plasmid to plant genome (Hooykaas and Schilperoort, 1984). Stachel *et al.* (1985) demonstrated the exudate containing specific substance, such as acetrosyringone (AS) and hydroxy-acetosyringone (OH-AS), from tobacco tissues could induce the activity of *vir* gene in Ti plasmid. Recent studies also indicated that some precursors of lignin such as coniferyl alcohol and sinapinic acid could also act as *vir* inducers (Stachel *et al.*, 1985, 1986; Melchers *et al.*, 1989; Spencer and Towers, 1988). These studies pointed out the *vir* inducers released from wounded tissues could play an important role for transformation. The low transformation efficiency have limited the usefulness of *Agrobacterium*-mediated transformation in soybean (Delzer *et al.*, 1990). The low transformation frequency in soybean might be due to the low level of phenolic compounds released from wounded plant tissues (Table 1). As comparing the contents of

phenolic compounds in tobacco, potato, and soybean, we found the AS contents of potato was higher than soybean (Table 1). This might explain why the different transformation efficiencies were existed between soybean and potato. Current studies also pointed out that the suitability of plant tissues for *Agrobacterium* transformation was dependent on different species, such as cotyledon was a good material for tomato, while stem segment seemed work well for *Brassica napus* (McCormick *et al.*, 1986; Fry *et al.*, 1987). We suspect that such different responses may be related to the amounts of the phenolic compounds and some unknown substances released from wounded target plant. In this study, AS was found to be unequally distributed in stem, leaf, and root of the same species (Table 1). The AS contents released from potato microtuber was higher than from leaf. This might explain why the tuber was more efficient for transformation than the stem or leaf tissue in potato (Chang and Chan, 1991; Sheerman and Bevan, 1988). In other words, the tissues which released more phenolic compounds could act as good materials for *Agrobacterium* infection.

For analyzing the phenolic compounds of potato suspension cultures by HPLC (Fig. 1), we found that SA and AS accumulated in the cultures were higher than other kinds of phenolic compounds (Table 2). We suspect that the decrease of SA and AS may be due to these accumulated phenolic compounds which were mobilized and changed into other substances. We also postulate that there might have other compounds rather than phenolic compounds present in young potato suspension cultures which are found to be beneficial for the induction of virulence of *Agrobacterium*. Vernade *et al.* (1988) indicated that glycine betaine could enhance the induction of *vir* gene by AS at low pH. The real function of these metabolites on the induction of *vir* gene of *Agrobacterium* needs further study.

Soybean explant (cultivar ADOC) treated with potato suspension cultures during infection by *Agrobacterium* afforded an effective way for forming transgenic calli and regenerating into plants from these calli. Results indicated natural phenolic compounds present in potato suspension cultures can effectively induce *vir* gene than pretreating bacteria with AS in the inoculation procedure. Similar result was observed by Bolton *et al.* (1986), and was pointed out that *vir* gene was more effectively induced in the infection stage than in the bacteria culture stage.

In this study, we use *in situ* localization to analyze the GUS activity in transgenic plants. The substrate, methyl umbelliferyl glucuronide, could be easily digested and formed 4-methyl umbelliferone which could produce fluorescence in gel under UV illumination. Thus, the GUS protein in transgenic plants can be clearly detected. From these results, we demonstrated that the addition of natural metabolites in the inoculation medium can increase the transformation frequency of soybean mediated by *Agrobacterium tumefaciens*.

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# 利用馬鈴薯懸浮細胞提升大豆轉殖效率之研究

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利用農桿腫瘤菌(*Agrobacterium tumefaciens*)轉殖大豆植株的轉殖效率通常不高，本研究之目的在探討大豆基因轉殖效率提升的方法。研究中所使用的基因載體爲帶有 GUS-neo 基因的嵌合型農桿腫瘤菌 C58C1 (pGV2260::NG1)及 C58C1 (pGV2260::NG3)兩菌系，並以此兩菌系感染大豆子葉。在感染過程中，將馬鈴薯懸浮細胞作爲感染液，以促使轉殖效率的提升。此外，本研究也利用添加 acetosyringone 於過夜培養的農桿菌培養液中作前處理，再利用此農桿菌感染大豆子葉，以和馬鈴薯懸浮細胞比較彼此對轉殖效率的促進效果。利用此方法所得到之轉殖癒合組織(因不同的大豆品種)顯著地提高了 54 至 70%。轉殖癒合組織及轉殖植株的 NPT II 酵素，已由 Dot-Blot 方法檢定出其活性。而轉殖植株中的 GUS 蛋白質，可藉由原位固定(*in situ* localization)膠體電泳法偵測出來。這些分析結果顯示外來的基因已轉殖進入大豆細胞中，並表現出其酵素活性。這些轉殖組織的證據更證實了此一轉殖效率增進方法的適用性。馬鈴薯懸浮細胞培養液中的植物酚類化合物，已由高壓液態色層分析儀檢定出其內含量及種類，在懸浮培養液中的高量 sinapinic acid 及 acetosyringone 正是促進轉殖效率的主要成分。