

## ***Agrobacterium tumefaciens*-Mediated Gene Transfer to Tea Plant (*Camellia sinensis*) Cells**

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### **Abstract**

Acetosyringone, which is one of the phenolic compounds produced by wounding plant tissues, actively induces the transfer of T-DNA from *Agrobacterium* to plant. To produce transgenic calli from tea plant (*Camellia sinensis*), leaf explants of the tea plant were co-cultivated with *Agrobacterium tumefaciens* LBA4404 harboring pB1121 under different acetosyringone concentration conditions. After the infection, these explants were placed on a medium containing 200 mg/L kanamycin and then resistant calli were selected. Although callus differentiation was not observed without acetosyringone at 10  $\mu$ M application, resistant calli were obtained from the explants treated with acetosyringone. In particular, the application of 500  $\mu$ M of acetosyringone promoted the production of resistant calli. When the fluorogenic assay was carried out to detect the GUS gene expression, one resistant callus showed the activation. In addition, PCR and PCR Southern blot analyses confirmed that the resistant calli were transformants.

**Discipline:** Biotechnology/Plant breeding/Tea industry

**Additional key words:** acetosyringone

### **Introduction**

Plant genetic transformation has been applied to many crop plants and some of the transgenic plants have been used commercially in recent years. Although some transformed woody plants also have been produced, such as apple<sup>6)</sup>, kiwi fruit<sup>17)</sup>, citrus<sup>11)</sup> and poplar<sup>1)</sup>, the number of woody plants species giving rise to transformants is limited. Since successful transformation is based on tissue culture techniques, regeneration from plant tissues or protoplasts, cannot be easily established for many woody plants.

Tea plant (*Camellia sinensis*), one of the important crops for beverage, is a woody plant. No stable technique of foreign gene introduction to the tea plant has been developed. For tea plant breeding, transformation technology will be a powerful tool. 'Yabukita', a leading variety of tea which covers 74% of the tea fields in Japan, is susceptible to several pathogens. To alleviate these shortcomings, genetic

engineering including a reliable transformation system is required. Several gene introduction methods, such as electroporation, PEG, bombardment and *Agrobacterium*-gene mediation, have been developed up to the present. In tea tissue culture, regeneration from protoplasts to a whole plant is difficult but possible from stem tissues or cotyledons. Although the ratio of infection is low, *Agrobacterium* can infect tea. In addition, *Agrobacterium*-gene transfer method does not require any expensive equipment, such as particle gun. Therefore, the *Agrobacterium*-gene mediated system is considered to be suitable for tea. At first we attempted to improve the ratio by the application of acetosyringone which is one of the phenolic compounds produced by wounding plant tissues and which induces the transfer of T-DNA from *Agrobacterium* to plants<sup>1,10,16)</sup>. In this study, we demonstrated that the use of acetosyringone is effective for *Agrobacterium*-gene transfer to the tea plant and enables to produce some transformed tea calli.

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## Materials and methods

### 1) Plant tissues and co-cultivation

Leaf was cut from *in vitro*-cultured tea plant which was a natural seedling of 'Sayamakaori' and prepared to approximately 10 × 10 mm fragments as an explant. The plant medium used in this study consisted of Murashige and Skoog<sup>12)</sup> standard 0.8% agar solid medium containing 3% sucrose, 0.5 mg/L IBA, 10 mg/L BA. Co-cultivation media with *Agrobacterium* contained acetosyringone at final concentrations of 10, 100 and 500 μM. *Agrobacterium tumefaciens* LBA4404 containing a binary vector pBI121 was used. The leaf explants were inoculated with *Agrobacterium* and cultured on the media for 2 days in the dark at 28°C. The leaf explants were washed with water, treated with carbenicillin (500 mg/L) to kill *Agrobacterium* and then transferred to a selective medium containing kanamycin (200 mg/L) and carbenicillin (500 mg/L). These explants were cultured under a 16 h/light, 8 h/dark, regime in a clean room controlled at 25°C and also transferred to fresh selective medium every 2 weeks.

### 2) Callus proliferation and evaluation

We developed a growth index (G.I.) indicated below and evaluated the degree of differentiation of callus from the explant 2 months after transfer to the selective medium.

- 0; Callus did not differentiate.
- 1; Callus differentiation only occurred at the periphery of the explants.
- 2; Callus covered about less than 30% of the explants.
- 3; Callus covered about more than 30% of the explants.

### 3) GUS fluorogenic assay

A binary vector plasmid (pBI121) contained a selective gene, NPTII, and a marker gene encoding the GUS controlled by the CaMV35S promoter. The fluorogenic assay was carried out as described by Jefferson<sup>7)</sup>. One resistant callus was ground with sand in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 10 mM EDTA, 0.1% Triton X-100). After centrifugation, 1 mM MUG buffer was added to the supernatant solution and the solution was incubated at 37°C for 2 h. A tube containing the solution was placed on an ultraviolet light box and the blue fluorescence was observed.

### 4) PCR and PCR Southern blot analyses

DNA was isolated from the resistant or non-resistant calli according to the method of Guillemaut<sup>3)</sup> with modifications and less than 10 ng DNA was used for the PCR template. Primer sequences were 5-GCATACGCTTGATCCGGCTACC-3 and 5-TGATATTTCGGCAAGCAGGCAT-3 for the NPTII gene and 5-GAACAACGAACTGAACTGGCA-3 and 5-CTAGTGCCTTGTCAGTTGC-3 for the GUS gene. Length of target DNA fragments of the NPTII or the GUS genes was 221 bp and 291 bp, respectively. Samples for enzymatic amplification were subjected to 30 repetitions of the following thermal cycle: 1 min at 95°C, 2 min at 55°C and 3 min at 72°C.

The amplified DNAs were electrophoresed in 0.8% agarose gel, stained with ethidium bromide and visualized by illumination with ultraviolet light. Then DNAs were transferred to Hybond N+ (Amersham) membranes and hybridized with fluorescein-labeled probes from the NPTII or the GUS DNA fragments. Hybridization was performed at 65°C overnight. After hybridization, the membranes were washed with 0.2 × SSC, 0.1% SDS at 65°C for 15 min twice.

Table 1. Effect of acetosyringone application on differentiation of resistant callus from tea leaf explants

Concentration of acetosyringone (μMm)	No <i>Agrobacterium</i> inoculation				<i>Agrobacterium</i> inoculation			
	Growth index				Growth index			
	0	1	2	3	0	1	2	3
0 <sup>a)</sup>	0	0	14	6				
0	10	0	0	0	20	0	0	0
10	4	6	0	0	17	3	0	0
100	4	6	0	0	9	7	4	0
500	4	6	0	0	5	9	2	5

a): No. of differentiated calli on a medium without antibiotics.

Chemical detection was carried out on X-ray film according to the manufacturers' manual (Amersham).

## Results

Results of callus differentiation from tea explants are shown in Table 1. Tea calli differentiated at the periphery of the explants and proliferated on the MS agar solid medium containing BA and IBA without antibiotics after 2 months. Fourteen ex-

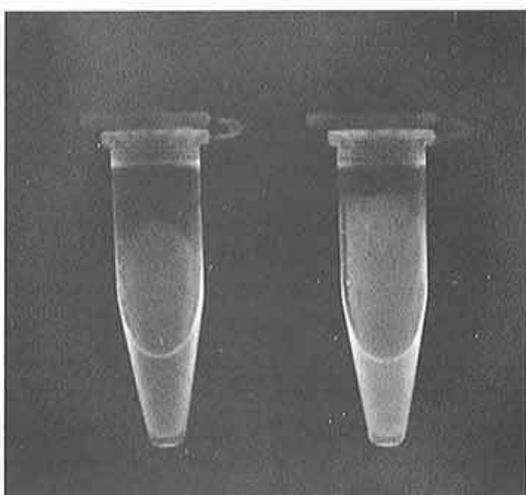


Fig. 1. Detection of GUS activity in resistant callus by fluorogenic assay  
Left: Non-resistant callus,  
Right: Resistant callus.

plants showed a growth index (G.I.) of 2 and calli from 6 explants also proliferated up to G.I. 3. On the other hand, calli on the selective medium containing 200 mg/L kanamycin did not grow even to G.I. 1. Therefore, the selection of kanamycin 200 mg/L was effective for the inhibition of normal differentiation or proliferation from the explants. These results also indicated that acetosyringone did not affect kanamycin selection since no relationship between the concentration of applied acetosyringone and the G.I. was observed.

None of the explants inoculated with *Agrobacterium* on the medium without acetosyringone or application of 10 mM induced calli on the selective medium. When 100 mM acetosyringone was used, 4 of the 20 explants induced G.I. 3 calli, and 5 calli in the 500 mM experiment could proliferate up to G.I. 3. These calli continued to grow on the selective medium and became green. Multiple transfers to the fresh medium were carried out to prevent the loss of antibiotics and contamination of *Agrobacterium*. Elimination may occur since *Agrobacterium* was not detected when part of the resistant calli was placed on LB agar medium. Therefore we attempted to use 5 calli in 500 mM for further analyses to determine whether these calli consisted of transformed cells.

To detect the expression of the GUS gene, we carried out a fluorogenic assay. The intense blue fluorescence of the resistant calli under UV light

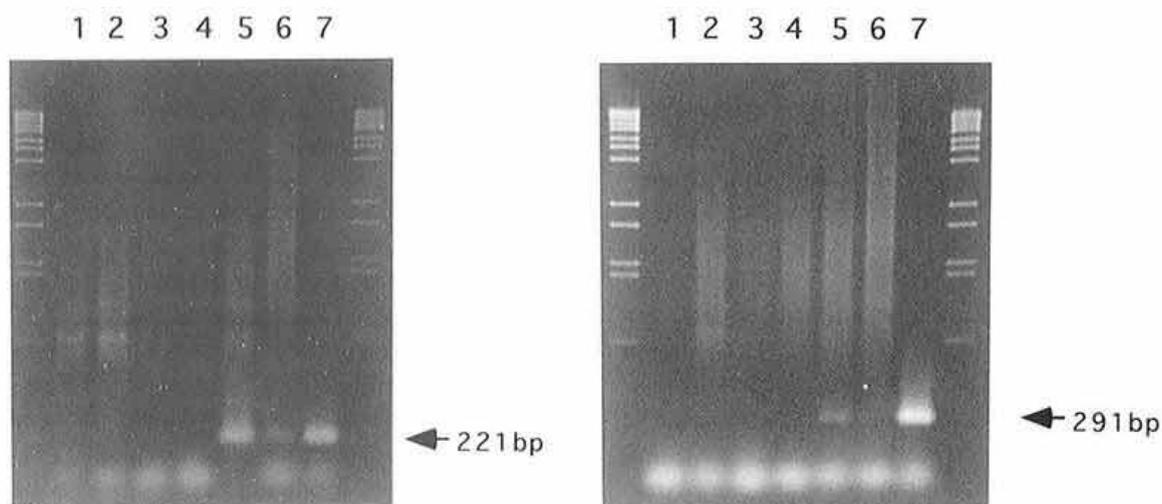


Fig. 2. Detection of amplified DNA fragments which are parts of NPTII (left) and GUS genes (right) in resistant callus genome by PCR analysis  
Arrows indicate length of target DNA amplified by PCR.  
Lane 1: non-resistant callus (as negative control).  
Lanes 2 to 6: resistant calli, calli No. 5, 4, 3, 2 and 1, respectively.  
Lane 7: pBI121 (as positive control).

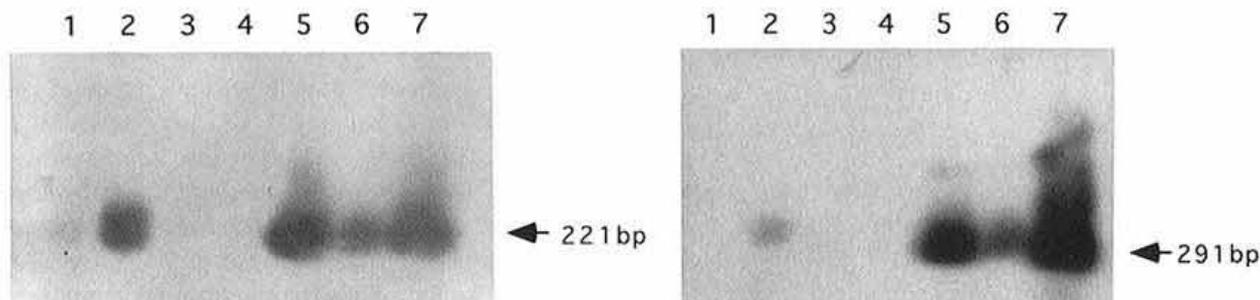


Fig. 3. Detection of hybridized DNA fragments with fluorescein-labelled probes, NPTII (left) and GUS genes (right) by Southern analysis of PCR products

Arrows indicate length of target DNA amplified by PCR.

Lane 1: Non-resistant callus (as negative control).

Lanes 2 to 6: Resistant calli, calli No. 5, 4, 3, 2 and 1, respectively.

Lane 7: pBI121 (as positive control).

was stronger than that of the negative control (Fig. 1). In the PCR analysis, 221 bp (NPTII) and 291 bp (GUS) DNA fragments were detected by staining callus No. 2 (Fig. 2, lane 5) with ethidium bromide as in the case of the positive control, pBI121, and a weak signal which showed the amplified 221 bp of NPTII was observed in callus No. 1 (lane 6). Hybridized fragments were observed not only in No. 1 and 2 calli but also in No. 5 callus (Fig. 3). Since the target DNA in a negative control experiment was not amplified, we assumed that calli No. 1, 2 and 5 consisted of transformed cells.

## Discussion

Several attempts to produce transgenic tea plants made until now had not been successful. The tea plant is, therefore, considered to be one of the recalcitrant plants for transformation. We selected the *Agrobacterium*-mediated gene transfer system as mentioned above. Tea plant is infected with *Agrobacterium* but shows a tendency to be resistant in the field, because the probability of observing natural crown galls on tea roots is low and even if it occurred, the damage would not reach an economic level. In the process of transformation through *Agrobacterium*, the low efficiency of the infection is a disadvantage which must be overcome. At first we tried to improve the ratio by using acetosyringone which had been employed to obtain transgenic plants, not only dicotyledonous plants such as *Arabidopsis*<sup>15)</sup>, sugar beet<sup>5)</sup> and tomato<sup>8)</sup>, efficiently but also monocotyledonous plants including rice<sup>4)</sup>. In these reports, acetosyringone was used at final concentrations ranging between 0.1 and 100  $\mu$ M.

The promotion of the expression of *Vir* genes began at 0.1 and reached a maximum level at 100  $\mu$ M<sup>16)</sup>. In this study the resistant calli only emerged by the application of higher concentrations of acetosyringone. Application of 100  $\mu$ M also promoted tea callus differentiation and callus grew up to G.I. 2. Since the G.I. 2 callus was resistant to kanamycin, it was considered that it consisted of transformed cells. These results indicated that the application of acetosyringone to the medium was very effective for tea cell transformation using leaf explants and the concentration for tea was higher than that of other plants.

One resistant callus showed the GUS gene expression, and PCR and Southern blot analyses revealed that 3 calli were transgenic. However, we could not confirm that 2 of the 5 resistant calli were transformants by PCR and PCR Southern analyses. The lack of amplification of the target DNA fragments of the 2 calli could not be explained, but may be due to the low concentration of template DNA under the current PCR thermal conditions.

Our final objective is to develop a technique for the introduction of foreign genes in the tea cells and production of transgenic tea plants by practical gene introduction. The tea plant can regenerate from cotyledon<sup>13)</sup> and stem<sup>9,14)</sup> in *in vitro* tissue culture. To produce a whole transgenic tea plant, it is necessary to combine 2 techniques for foreign gene introduction and regeneration from transgenic cells. We demonstrated that acetosyringone application was effective in the production of transgenic tea cells. These results will be applied in a following study to produce whole transgenic tea plants.

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