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Transformation of Tobacco Cultured Cell by Particle Bombardment: Expression of Phenylalanine Ammonia-lyase Gene in Transgenic Tobacco Cells

Nobuo NAGAI*, Kuninori YANAGISAWA*, Kazuko MIZUNO*, Hirofumi IMURA*, Goro TAGUCHI*, Makoto SHIMOSAKA*, Daisuke SHIBATA** and Mitsuo OKAZAKI*

* *Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University*, Ueda, Nagano 386, Japan

** *Mitsui Plant Biotechnology Research Institute*, Tsukuba, Ibaraki 305, Japan

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The plasmid DNA containing a NPTII as a selectable marker gene and a tobacco PAL cDNA combined with the CaMV 35S promoter and NOS terminator was transformed into tobacco cells by a particle bombardment device based on flowing helium. The most efficient transformation was achieved when the amount of DNA-coated tungsten particle was 0.2 mg per projectile, the amount of plasmid DNA was 4 μg/mg of tungsten particles, the helium pressure accelerated was 3 kg/cm^2, and the distance between the sample and the projectile was 15 cm. Genetic analysis of kanamycin-resistant transformants obtained showed that the PAL cDNA construct integrated into the genome of tobacco cells. PAL activity of the transformant increased almost 4-fold and scopoletin content increased more than 2-fold as compared to nontransformed cells.

Introduction

Phenylalanine ammonia-lyase(PAL; L-phenylalanine ammonialyase, EC 4.3.1.5) catalyzes the first step in the synthetic pathway for a diverse array of plant natural products having phenylpropane skeleton. We had already reported that the addition of kinetin in tobacco cell (*Nicotiana tabacum* L. Bright Yellow T-13) culture increased PAL activity (about 2-fold), and caused an accumulation of a high level of scopoletin[1]. To clarify the mechanism of this kinetin-mediated PAL induction, we had cloned a full-length PAL cDNA (2,546-bp full-length PAL cDNA containing a 2,136-bp open reading frame, a 204-bp 5′-noncoding region, and a 206-bp 3′-noncoding region containing a 19-bp polyA)[2].

In order to study a regulatory mechanism in phenylpropanoid synthetic pathway, and to obtain a tobacco cell capable of forming a higher level of scopoletin by molecular breeding, we tried to introduce a PAL cDNA into tobacco cells using a particle bombardment device based on flowing helium[3,4].

Materials and Methods

1. *Tobacco callus and plasmid DNA*

A habituated *Nicotiana tabacum* L. "Bright Yellow" T-13 callus was maintained on hormone-free Murashige and Skoog (MS) agar medium[5] wish 4 weeks interval subculturing. The suspension cultures were obtained by transferring the cell clumps to MS liquid medium, and cultured on a

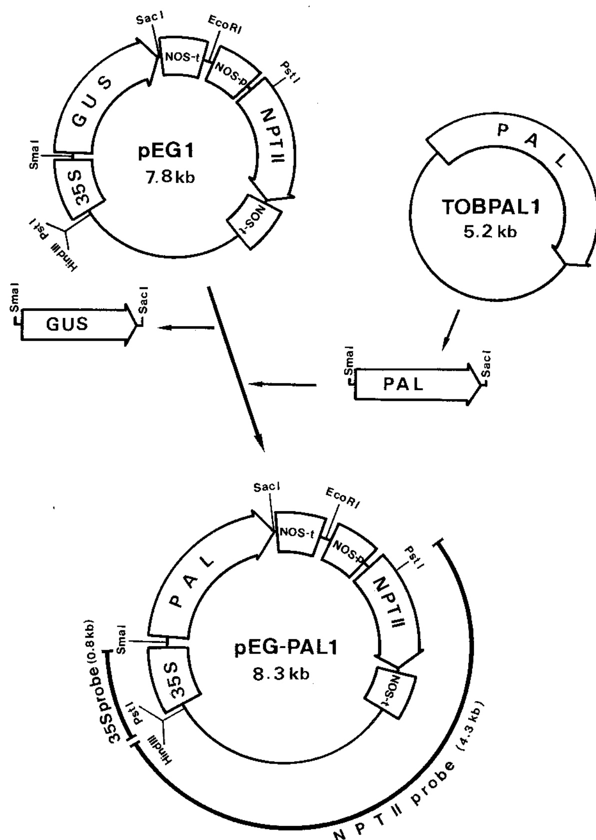

Fig. 1 Construction of a plasmid vector pEG-PAL 1 used for particle bombardment. A NPT II probe and a 35 S probe which were used in Southern blot analysis were indicated. Abbreviation: GUS, β-glucuronidase; NOS, nopaline synthase; NPT II, neomycin phosphotransferase; PAL, phenylalanine ammonia-lyase.

rotary shaker (100 rpm) at 30°C for 2 weeks, and these suspension cell cultures were used in subsequent investigations.

The DNA construct used for transformation contained tobacco PAL cDNA[2] or β-glucuronidase (GUS) gene in a control experiment. Chimeric plasmid DNA pEG1 (a gift from Tetsu Kawazu, Kameyama Breeding Station, New Oji Institute for Forest Tree Improvement, New Oji Paper Co. Ltd.) [7. 8 kilobase (kb)], which contains the promoter-less GUS gene linked with the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. The pEG1 also contained the neomycin phosphotransferase (NPTII) gene under the control of NOS promoter and NOS terminator as a selectable marker (**Fig. 1**). The plasmid pEG-PAL1 was constructed by replacing the GUS gene in pEG1 with the tobacco PAL cDNA fragment containing the whole protein coding region (**Fig. 1**).

2. *DNA transfer and selection of transformants*

i) Transformation of DNA into intact tobacco cells

The tobacco callus (about 500 mg-wet weight) was placed on a Whatman no. 1 filter paper (3. 0 cm in diameter), or the suspension cultured cell was evenly distributed over the surface of filter paper with the aid of a Buchner funnel. The filter paper with cells was placed on MS medium solidified with agarose (8. 5 g/liter) in a Petri dish (6. 0 cm in diameter). Cells were then bombarded in a vacuum (60 mmHg) with tungsten particles (GTE Sylvania, average diameter of 1. 3 μm) to which plasmid DNA was previously adsorbed[4]. The particle bombardment device used in this experiment

has been detailed[3,4].

ii) Detection of GUS expression in transformed cells

Following bombardment, the filter papers with cells were transferred onto fresh MS medium solidified with agarose (8.5 g/liter), and incubated for 48 hr at 27°C. The resulting filter paper was then transferred onto a Petri dish previously contained 300 μl of the GUS substrate mixture, so that the filter paper adsorbed the substrate mixture evenly over its entire area. The substrate mixture consisted of 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1.9 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc, the substrate of GUS) (Wako Pure Chemical Industries), and 0.3% (v/v) Triton X-100. Then cells were incubated for 24 hr at 37°C, successively added with 2 ml of 70% (v/v) ethanol to stop the reaction and to maintain an aseptic condition. GUS-expressing cells were detected microscopically by the distinct blue color that developed within their interior as a result of enzymatic cleavage of X-Gluc.

iii) Selection of stable transformants

Following bombardment, the filter papers with cells were transferred onto fresh MS medium solidified with agarose (8.5 g/liter) and incubated for 5 days at 27°C. Then cells were plated on agar-solidified MS medium containing kanamycin (100 μg/ml). Kanamycin-resistant cells were identified after 1 month of incubation.

3. *Detection of PAL expression in transformed cells*

i) Southern blot analysis

For Southern blot analysis, total genomic DNA prepared from transformants were digested with *Eco*RI and *Hin*dIII. The resulting DNA fragments were fractionated by agarose (1%) gel electrophoresis, followed by blotting to a nylon membrane (Gene Screen Plus, Du Pont). The 4.3-kb *Pst*I fragment containing the NPTII gene and the 0.8-kb *Hin*dIII-*Sma*I fragment containing CaMV 35S promoter from pEG1 were ^{32}P-labeled by random priming (Multiprime Labeling System, Amersham, Japan), and used as a probe in Southern blot analysis (**Fig. 1**). The prehybridization was done at 42°C for 30 min. in a hybridization buffer consisting of 50% (v/v) formamide, 1% (w/v) SDS, 2×SSC (0.15 M NaCl, 15 mM sodium citrate), and 10% dextran sulfate. The hybridization was done at 42°C for 16hr in the same solution to which 50 μg/ml of denatured salmon sperm DNA and the ^{32}P-labeled probe had been added. The membranes were washed at 42°C for 20 min. twice in 2×SSC, 1% SDS, and once in 0.2×SSC, 1% SDS, and autoradiographed on X-ray film.

ii) Western blot analysis

Total proteins extracted from cells with 50 mM borate buffer (pH 8.8) containing 5 mM 2-mercaptoethanol were analysed by SDS-PAGE by the method of Laemmli[6]. The PAL protein was detected by Western blot analysis using rabbit anti-tobacco PAL-IgG[7] as a first antibody and goat anti-rabbit IgG conjugated with peroxidase as the second antibody by the method of Remick *et al*[8,9].

iii) Assay of PAL activity and scopoletin formation

PAL activity and scopoletin content in cells were determined as described previously[1]. PAL activity was determined from the rate of the formation of t-cinnamic acid, which was spectrophotometrically measured by the increase in absorbance at 290 nm at 30°C. One unit was defined as the amount forming 1 μmol t-cinnamic acid per minute under the assay conditions.

Results and Discussion

At first, in order to determine the optimum conditions for use of a particle bombardment device, we transformed tobacco cells by the device with DNA (pEG1: 35S-GUS)-coated tungsten particles

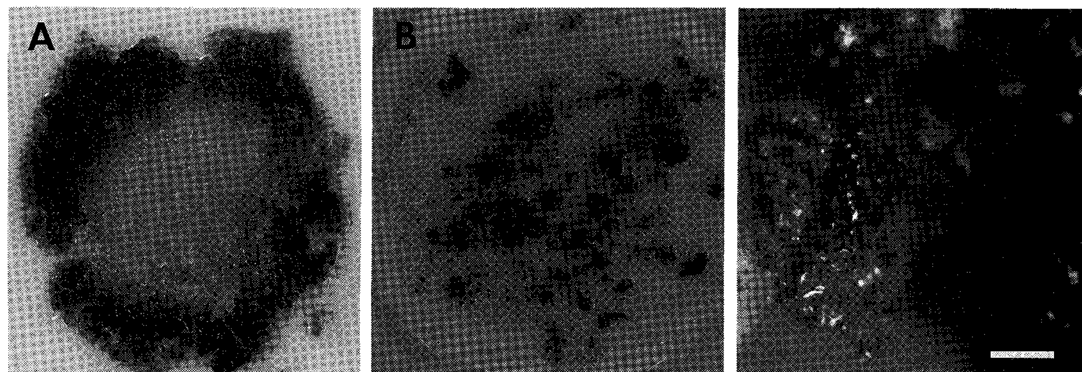

Fig. 2 Expression of GUS gene in tobacco cells transformed with pEG 1.
Tobacco callus (A), suspension culture cells (B) and a microphotograph of callus (C) expressing GUS activity two days after bombardment. The expression of the GUS gene produced intense dark color spots. Scale bar of the panel C is 1 mm.

Table 1. Transient expression of GUS gene in transformants.

Target tissue	Target distance (cm)	Transient expression: number of blue foci
Suspension	4	16
cultures (2×10^6 cells)	15	42
	20	32
Callus	4	4
(1 g fresh weight)	15	450
	20	66

and judged the result using transient assay of GUS. Typical results of the GUS assay for tobacco callus and suspension cultured cells that had been bombarded with tungsten particles coated with plasmid pEG1 were shown in **Fig. 2**. The appearance of a large number of dark spots indicated that GUS-expressing cells were present in the callus of the suspension cultured cells, however, dark spots were not present at all in the control tobacco cells bombarded with non-coated tungsten particles (data not shown). These results showed that particle bombardment provided a successful delivery of plasmid DNA into tobacco cells and the expression of the introduced gene. **Table 1** shows the effect of the distance between the sample and the projectile on transient GUS expression (number of blue spots) in tobacco cells. The maximum number of blue spots was obtained at the distance of 15 cm; 450 foci per 1 g-fresh weight in the callus and 42 foci per 2×10^6 cells in the suspension culture (average of 2 independents). These data were lower than the data of transformation of tobacco cells (*Nicotiana tabacum* L. cv. Bright Yellow-2)[10] and pollen (*Nicotiana tabacum* L. cv. Samsun)[11] by a pneumatic particle gun. An examination of other conditions led to the optimum bombardment conditions: the amount of DNA-coated tungsten particles, 0.2 mg per projectile; the amount of plasmid DNA, 4 μg/mg of tungsten particles; and the helium gas pressure of the projectile, 3 kg/cm^2.

To determine whether stable transformants could be obtained from callus or suspension cultures, cells were bombarded with tungsten particles coated with the plasmid pEG-PAL1 containing a NPTII gene as a selectable marker (**Fig. 1**), and then transformants were selected on kanamycin-containing medium. Six independent kanamycin-resistant calli were obtained from callus transformed with pEG-PAL1. To obtain evidence for stable transformation, total genomic DNA was extracted from kanamycin-resistant callus and control callus, digested with *Eco*RI, and analyzed by

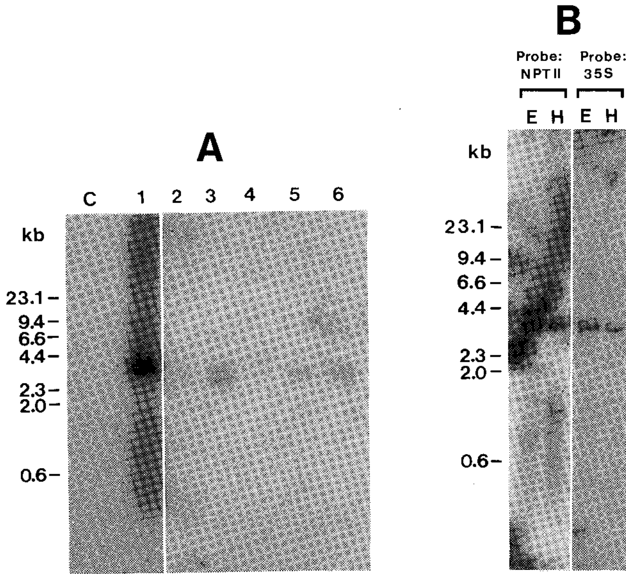

Fig. 3 Southern blot analysis of transformed tobacco cells.
A: Genomic DNA was digested with *Eco*RI and hybridized with a ^{32}P-labeled 4. 3-kb *Pst* I fragment containing the NPT II gene (NPT II probe). Lane C, DNA from nontransformed control cells; lane 1-6, six independently transformed clones.
B: Genomic DNA of clone # 1 was digested with *Eco*RI (lane E) and *Hin*dIII (lane H) and hybridized with a NPT II probe (described above) or a ^{32}P-labeled 0. 8-kb *Hin*dIII -*Sma* I fragment containing CaMV 35 S promoter (35 S probe).

Southern blot hybridization using a NPTII gene as a probe. Every kanamycin-resistant clone tested showed one or two hybridizing bands that were larger than 3. 0-kb in size, whereas nonbombarded callus did not show any signal (**Fig. 3-A**). These results indicated that the plasmid pEG-PAL1 DNA was introduced into the genome of tobacco cells. Out of the six kanamycin-resistant clones, one callus (# 1) was chosen and further analyzed, since this clone showed the strongest band in the autoradiogram. As shown **Fig. 3-B**, a single signal (lane H hybridized with a NPTII probe and lane E, H hybridized with 35S probe) or double signals (lane E hybridized with a NPTII probe) that were larger than 3. 0-kb in size was detected by Southern blot analysis. These results suggested that the entire NPTII gene and 35S promoter region of the plasmid pEG-PAL1 DNA was introduced into the genome of clone # 1. It was also suggested that several copies of pEG-PAL1 might be introduced into the genome of clone # 1 by comparing the densities of signals with copy marker bands used in the same Southern blot analysis (data not shown). Further, we analyzed by Southern blot hybridization using a tobacco PAL cDNA as a probe. There were difficulties in the analysis because the Southern blot analysis was complicated by many signals of an endogenous PAL gene.

To determine the expression of the introduced PAL cDNA genes in the clone # 1 cells, PAL activity and scopoletin content were assayed. PAL activity increased almost 4-fold and scopoletin content increased more than 2-fold in clone # 1 as compared to the nontransformed control cells (average of 2 independents) (**Table 2**). Immunoblotting analysis revealed a single band reacted with anti-PAL antibody at the position corresponding to the subunit (MW: 74, 000 Da) of tobacco PAL (PAL enzyme protein consisted of four homogeneous subunits.) (**Fig. 4**). Several minor proteins reacted nonspecifically. Comparing the intensity of protein bands, the PAL reacted with anti-PAL antibody revealed an increase of PAL content in clone # 1. These results indicated that

Table 2. PAL activity and scopoletin content of transformed tobacco cells.

	PAL activity*1 (mU/mg protein)	Scopoletin content*2 (μmol/g Dry wt.)
Nontransformed cells	1.0	0.26
Transformed clone #1	4.4	0.62

*1 PAL activity was assayed using cells cultured for 5 days. Average of two independents.

*2 L-Phenylalanine (precursor) (0.3 mg/ml) and casamino acids (3 mg/ml) were added on the 11 th day and the cells were harvested on the 14 th day after inoculation. Average of two independents.

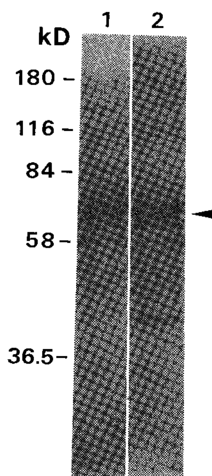

Fig. 4 Western blot analysis of PAL in transformed and nontransformed tobacco cells.
Cell extract was obtained by homogenizing cells as described in Materials and Methods. The resulting samples (100 ng protein each) were electrophoresed on a 10% SDS-polyacrylamide gel. After transferring the fractionated proteins on the polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories), immunoblotting with rabbit anti-PAL IgG was performed. Lane 1, nontransformed control cells; lane 2, transformed clone #1. The arrow indicated the position of the subunit of tobacco PAL (MW: 74,000 Da).

several copies of tobacco PAL cDNA were introduced into the genome of clone #1 cells and expressed.

Elkind *et al.* reported that the introduction of a heterologous PAL (genomic DNA) (from *Phaseolus vulgaris*) into tobacco cells (*Nicotiana tabacum* cv. Xanthi) resulted in a decrease of PAL activity and a perturbation of phenylpropanoid biosynthesis[12]. In this study, PAL activity and the formation of scopoletin in transgenic tobacco cells were increased by the transformation of a homologous PAL gene indicating the possibility of breeding tobacco cells capable for a higher level of phenylpropanoid biosynthesis by gene dosage effect.

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《和文要約》

パーティクルガンによるタバコ培養細胞への遺伝子導入:
phenylalanine ammonia-lyase (PAL) cDNA の導入と遺伝子発現

永井伸夫*・柳沢訓賀*・水野和子*・井村洋章*・田口悟朗*・下坂　誠*・柴田大輔**・岡崎光雄*

* 信州大学繊維学部 応用生物科学科
** 三井業際植物バイオ研究所

ヘリウムガス圧式パーティクルガンにより，タバコ培養細胞の PALcDNA を同細胞へ再導入し，形質転換体を得た．タバコ培養細胞の PALcDNA をカリフラワーモザイクウイルス (CaMV) の 35S プロモーターとノパリン合成酵素 (NOS) 遺伝子のターミネーターに接続し，ネオマイシンホスホトランスフェラーゼ II (NPTII) 遺伝子とともにタバコ培養細胞へ導入し，形質転換体をカナマイシン培地で選抜した．また，パーティクルガンによる遺伝子導入の最適条件としては，加速圧力 3 kg/cm^2，0.2 mg タングステン粒子/プロジェクタイル，4 μg DNA/mg タングステン粒子，プロジェクタイルからサンプルまでの距離は 15 cm であった．そして得られた形質転換体の中には，非形質転換体と比較して，PAL 酵素活性で 4 倍，スコポレチン生成量で 2 倍以上のものが確認された．