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Expression of Modified Green Fluorescent Protein in Suspension Culture of *Taxus cuspidata*

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Abstract The suspension cells of *Taxus cuspidata* were transformed with *Agrobacterium tumefaciens* harboring binary vector pCAMBIA1302 encoding *mgfp*. Transient transfection efficiency was compared by using the fluoremetric measurement. The transient transfection efficiency was improved by transformation with DMSO and/or sonication treatment. Optimum conditions for DMSO and sonication treatment were 3% and 30 sec, respectively. Selection and maintenance of transformed cells were continued for 3 months. An insertion of the *mgfp* gene in transformed cells was detected by PCR and an expression of GFP confirmed by the western blot analysis.

Key words: *Taxus cuspidata*, GFP, *Agrobacterium*, transformation

Cell cultures of *Taxus* spp. are a potential alternative source for the production of paclitaxel, which is known to be an effective anticancer drug. Despite of many studies on the production of paclitaxel using the suspension culture of *Taxus* spp., the low productivity still remains to be one of the major barriers for its commercial production. As more information becomes available in relations to the genes which are involved in the biosynthetic pathway of secondary metabolite production, metabolic engineering by recombinant DNA technology will serve as a practical tool to improve the yield of anticancer agents from the plant cell culture. The possibility of introducing a foreign gene into *Taxus* spp. was first demonstrated by Han *et al.* [3] using *Agrobacterium*-mediated transformation. Transient GUS expression in zygotic embryos of *Taxus brevifolia* using a direct DNA uptake method was also reported [5]. However, an optimum condition for *Agrobacterium*-mediated transformation of *Taxus* spp. has not been fully optimized as of yet. In this work, we investigated the *Agrobacterium*-mediated transformation of a suspension culture of *T. cuspidata*.

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Suspension cultures of *T. cuspidata* were established from young stem segments of yew trees grown at Suwon, Korea, as described previously [7]. Cell suspension was maintained at 110 rpm and 26°C under a dark condition using a modified Gamborg's B5 medium supplemented with 0.5 g/l casein hydrolyzates, 4 mg/l 2,4-dichlorophenoxyacetic acid, and 30 g/l sucrose.

For transformation of suspension cells, the disarmed *Agrobacterium tumefacians* strain LBA4404 containing the binary vector pCAMBIA1302 encoding *mgfp5* (MRC, U.K.) was used. *Agrobacterium* cells were cultured overnight at 28°C on a Luria Bertani (LB) medium containing rifampicin 50 mg/l, kanamycin 50 mg/l, harvested by centrifugation and resuspended in an induction medium (mB5 supplemented with 10 g/l glucose and 100 μM acetosyringone, and adjusted to pH 5.2). The suspension cells of *T. cuspidata* were then co-cultivated with *Agrobacterium* cells at 25°C and placed in a dark condition for 3 days. After co-cultivation, cells were washed three times with sterilized distilled water and then transferred to a selection medium with 250 mg/l cefotaxime and 10 mg/l hygromycin. After two days, some of the transfected cells were harvested for the analysis of transient expression of GFP. All the transfected cells remained were cultivated in a selection medium for 4 weeks. Thereafter, actively growing cells were selected and maintained for three months.

For measuring the GFP fluorescence, the cells (0.5 g fresh weight) which had been harvested in liquid N_2 were grounded with a pestle and a pre-chilled mortar in 2 volumes of ice-cold extraction buffer [50 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM dithiothreitol, 20% (v/v) glycerol]. The extract was sonicated for 2 min and centrifuged for 15 min at 14,000 rpm to precipitate debris and the supernatant was collected. The fluorescence of the protein fraction was measured at 515 nm using a 460 nm excitation in a Turner Digital Fluorometer (Model 450, Barnstead/Thermolyne, Dubuque, U.S.A.). All the transient transfection efficiencies were compared by using the relative fluorescence intensity.

The GFP expression in stably transformed cells was also confirmed by western blot analysis, described as follows. SDS-PAGE was carried out on a 12% (w/v) SDS-polyacrylamide gels according to Laemmli [4]. Proteins separated in a gel were transferred to nitrocellulose membrane in a transfer buffer. Immunodetection was conducted with GFP polyclonal antibody (Clontech, Palo Alto, U.S.A.) at a 1:1,000 dilution in TBS [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 3% (w/v) skim milk]. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich, St. Louis, U.S.A.) was used at a 1:1,000 dilution in TBS and it was detected by using NBT/BCIP.

Experiments were made to find out the effect of acetosyringone in relations to the transient transfection efficiency since acetosyringone would often be used for inducing virulence in *Agrobacterium* strains [8]. Relative fluorescence increased abruptly at 50 μM. GFP expression reached its maximum at 200 μM of acetosyringone (Fig. 1). This shows that acetosyringone, a natural phenolic compound, could be used to improve efficiency for *Agrobacterium*-mediated transformation.

Effect of pH on transient transfection efficiency was also examined. As shown in Fig. 2, transient transfection efficiency showed the best at pH 5.2. This result indicates that the induction of virulence of *Agrobacterium* could be dependent on the pH condition.

Suspension cells of *T. cuspidata* are very sensitive to the *Agrobacterium* invasion. In *Agrobacterium*-mediated transformation, the density and co-cultivation time of *Agrobacterium* have been reported to be critical for the plant cell viability [1]. As shown in Fig. 3, relative GFP fluorescence in cells after cultivation for 1 day remained relatively constant for cell densities which were higher than 0.5×10^9 cells/ml. However, a relative fluorescence for a co-cultivation for 3 days was higher than those for co-cultivation for 1-2 days with respect to the whole range of cell densities. This means that the infection density of *Agrobacterium* could be reduced to 0.5×10^9 cells/ml, and the co-cultivation time of 3 days is better for *Agrobacterium*-mediated transformation of *T. cuspidata*.

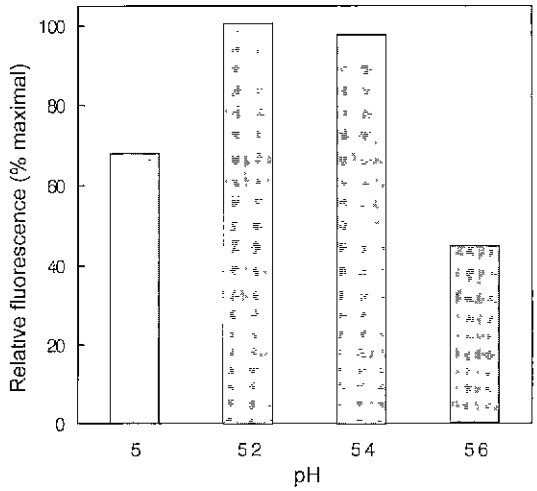

Fig. 2. Effect of pH on transient transfection efficiency.

Plant transformation efficiency is limited by the host-susceptibility associated with the use of *Agrobacterium*-mediated transformation [6]. Therefore, the chemical and physical treatment affecting the membrane structure of plant cells could be applied to alter the susceptibility of plant cells. Since DMSO has often been used in permeabilizing plant cell membrane [2], we examined the effect of DMSO treatment (for 24 h) on a transient transfection efficiency of *T. cuspidata* cells. As shown in Fig. 4, relative fluorescence increased up to 5% DMSO. The exact mechanisms of DMSO for enhancing transient transfection efficiency remain unclear; however, this might

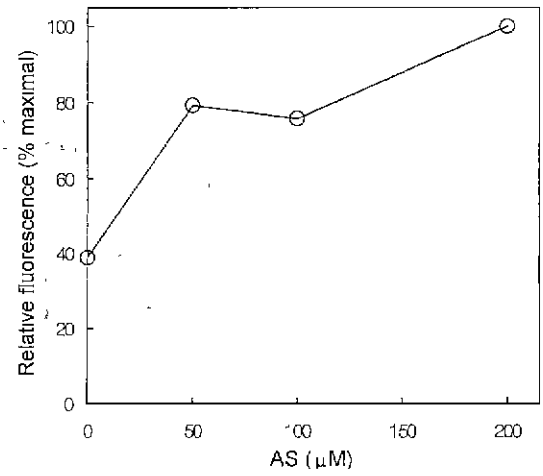

Fig. 1. Effect of acetosyringone on transient transfection efficiency.

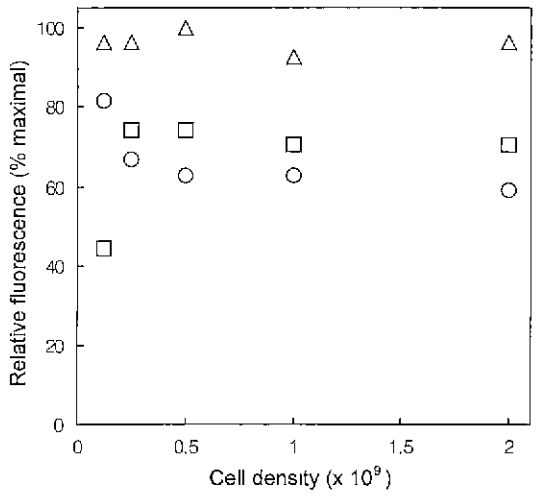

Fig. 3. Effect of *Agrobacterium* density and co-cultivation time on transient transfection efficiency; □, 1 day; ○, 2 day; △, 3 day.

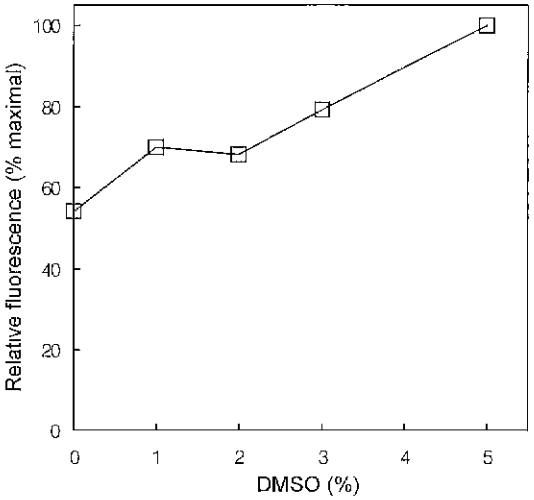

Fig. 4. Effect of DMSO on transient transfection efficiency.

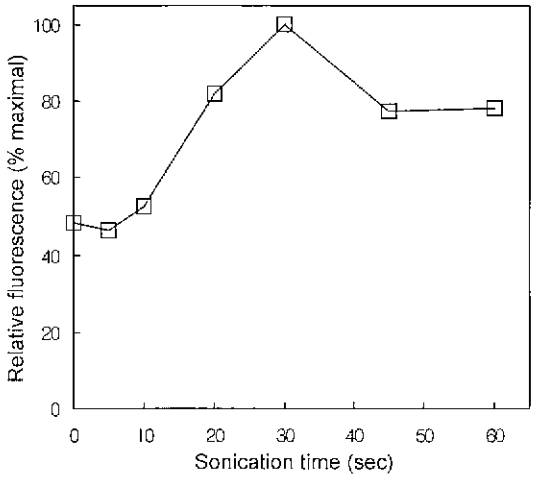

Fig. 5. Effect of sonication time on transient transfection efficiency.

be due to an alteration of membrane permeability by DMSO treatment.

The effect of physical pre-treatment, like sonication, on transfection efficiency for suspension cells of *T. cuspidata* was also investigated. The mixture of *T. cuspidata* and *Agrobacterium* cells in a 50-ml conical tube were sonicated and thereafter co-cultivated for 3 days. The transfection efficiency increased up to 30 sec and then decreased. The relative fluorescence was maximal at 30 sec of sonication. The relative fluorescence was improved as much as 200% by using the sonication-assisted transformation. We speculate that microwounds of plant cells and tissue induced by sonication could facilitate the infection of plant tissues by *Agrobacterium* [9].

The transformed suspension cells were maintained for 3 months. Thereafter, the GFP expression was analyzed using the western blot analysis. As shown in Fig. 6.

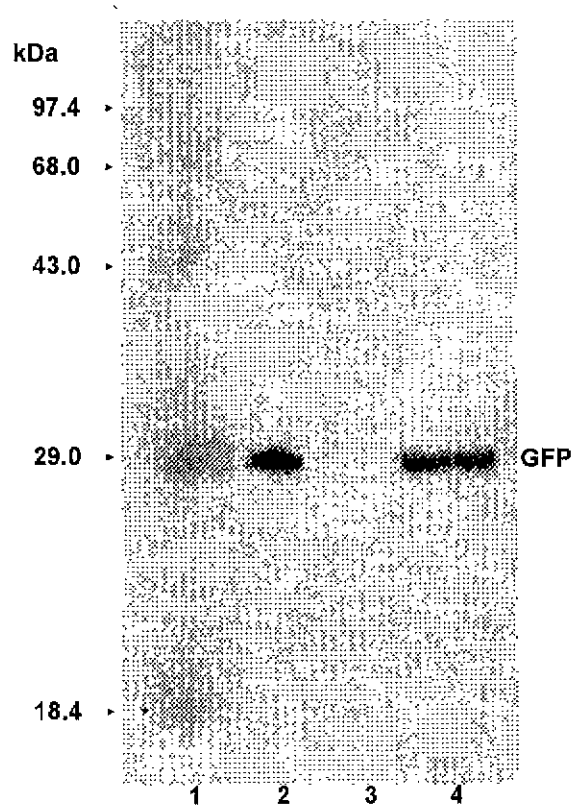

Fig. 6. Western analysis of GFP.
Lane 1: molecular marker; Lane 2: GFP standard; Lane 3: non-transformed cells; Lane 4: transformed cells

recombinant GFP in transformed cells was found in the intracellular fraction with a molecular weight of 30–32 kDa. The GFP expression was not detected in the extracellular fraction of transformed cells (data not shown). The molecular weight of newly expressed GFP in *T. cuspidata* cells was slightly larger than that of the wild-type GFP standard, probably because pCAMBIA1302 construction contains nucleotide sequences of modified GFP and his-tag terminal.

In this work, we investigated several parameters affecting transformation efficiency in *Agrobacterium*-mediated transformation of *T. cuspidata* cells. Our results indicate that transformation efficiency could be improved by optimizing environmental parameters such as acetosyringone, pH, cell density, DMSO, and sonication. We also demonstrated that GFP from *Aequorea victoria* can be successfully expressed in *T. cuspidata* cells and that GFP can be a useful reporter for analyzing the gene expression.

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