Preliminary

Expression System for Foreign Genes using Protoplasts from *Liriodendron tulipifera* L. Suspension Culture*[1]

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Introduction

The traditional breeding in woody plants hardly improves their traits because of their long generation time. Genetic engineering may provide a powerful tool to improve their traits that could be changed in a short time. To achieve the engineering, we need genetically stable and homogeneous plant materials. *In vitro* culture system of woody plants would be expected to be good for material supply, genetic homogeneity and stability. Yellow-poplar (*Liriodendron tulipifera* L.) is a fast growing tree that is useful for wood and pulp products. Direct gene transfer of yellow-poplar has, already, been investigated by microprojectile bombardment[1].

Electroporation is one of the methods introducing genes directly into protoplasts. The method has permitted high efficiency for the transformation and also been applied on the analysis of gene regulations.

In this report, we describe *in vitro* culture and electroporation-mediated gene transfer systems of yellow-poplar. In addition, we report the attempt to test whether a mammal gene, goat growth hormone (gGH), is expressed in this plant system.

Materials and Methods

Cell suspension culture of yellow-poplar (*Liriodendron tulipifera* L.) was initiated from shoot apex-induced callus. The cells were grown in Murashige and Skoog (MS) medium[2]

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supplemented with 3.0% sucrose, 0.1% NZ-amine, 5.0 μM of 2, 4-D and 0.5 μM of 6-BA. For protoplast isolation, cells were harvested from 6 days old suspension cultures and were incubated in the enzyme solution, pH 5.7 containing 2.0% Cellulase Onozuka RS, 0.1% Pectolyase Y-23, 0.5 mM $CaCl_2$ and 0.5 M mannitol at 30°C for 2 h. The isolated protoplasts were suspended in 0.5 M mannitol containing 0.5 mM $MgCl_2$. Protoplast viability was determined by staining cells with fluorescein diacetate.

A 0.5-ml aliquot of protoplast suspension (1.5×10^5 cells) was placed in an electroporation cuvette with a 0.4-cm space between the electrodes (BRL Life Technology). After addition of plasmid DNA (foreign gene) and cation, protoplast samples were kept on ice for 10 min before being subjected to electroporation at various pulse length and voltage by Cell-Porator Electroporation apparatus (BRL Life Technology). Electroporated samples were diluted with 2.5 ml of MS medium supplemented with 5.0 μM of 2, 4-D, 0.5 μM of 6-BA and 0.5 M mannitol and cultured in dark at 28°C.

Two plasmids were used as foreign genes in this experiment. Plasmid pFF19G[3] contains bacterial β-glucronidase (GUS) gene, which was expressed under the control of CaMV 35S promoter. The other plasmid pFF19GH01 was constructed by replacing the GUS gene in pFF19G with the cloned cDNA fragment encoding goat growth hormone (gGH)[4]. The gGH gene was, therefore, flanked with the 35S promoter and 35S poly A signal terminator.

After being cultured 32 h, protoplasts were collected by centrifugation, and GUS activity in cells was determined according to the method of Jefferson[5] to optimize transient gene expression. For detection of gGH gene expression, total RNA and proteins extracted from cells was analyzed by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) and Western blotting, respectively.

Results and Discussion

White and friable callus were formed by shoot apex explants on agar plate in a month. Cell suspension cultures were subsequently obtained by incubating the callus in a liquid medium. Fresh weight of the cells was increased ten times during 2 weeks. At the early log phase of cell growth, most of the cells were converted enzymatically to protoplasts, and the yield was $6–8\times10^7$ protoplasts/gfw of cell. Freshly isolated protoplasts were about 50 μm in diameter and of high viability, more than 85%.

In order to develop a transient expression system for foreign gene, isolated protoplasts were transfected with bacterial GUS gene as a reporter by electroporation. GUS activity in the transfected cells reached the highest level (5.8 nmoles 4-MU/min/1.5×10^5 protoplasts) at 32 h after electroporation and then decreased showing typical transient gene expression. Optimal conditions for transient GUS gene expression were as follows : (a) The electric field

strength and pulse length for maximum GUS activity were 700 V/cm and 6.6 ms, respectively. (b) Concentration of foreign plasmid DNA was at 20 μg/ml. (c) 0.5 mM Mg^{2+} in electroporation solution was effective. (d) Protoplasts concentration was 1.5×10^5 protoplasts/ml.

Subsequently, to confirm applicability of the GUS expression system of yellow-poplar, we introduced a goat growth hormone (gGH) gene to protoplasts under the optimal condition described above. The result of RT-PCR analysis revealed that the specific transcript for gGH gene was produced in the cells transfected with plasmid pFF19GH01 harboring gGH gene. Furthermore, Western blot analysis of total protein from the cells showed that 22 kDa gGH protein was also synthesized in the electroporated cells. These results showed that gGH gene was expressed at the transcription and translation level in the yellow-poplar cells.

In this study, we developed efficient transient gene expression system using protoplasts of yellow-poplar. The system should provide an important basic step towards researching the regulation of gene expression and producing new woody plants.

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