INTRODUCTION OF FOREIGN DNA INTO YELLOW-POPLAR PROTOPLASTS BY ELECTROPORATION

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Abstract. Plantlets of yellow-poplar can be regenerated from protoplasts by somatic embryogenesis. This culture system could allow the production of genetically altered trees following DNA uptake into protoplasts. Two bacterial marker genes, ß-glucuronidase (GUS) and neomycin phosphotransferase (NPT II), are being used to monitor yellow-poplar transformation. Plasmids bearing one or both of these genes were introduced into yellow-poplar protoplasts by electroporation or polyethylene glycol-mediated uptake. The protoplasts had been isolated from embryogenic suspension cultures at different timepoints after transfer to fresh medium. In addition to the physical parameters of electroporation, such as voltage, capacitance, and pulse length, an important factor in delivery of DNA into cell nuclei was the interval between subculture and exposure to DNA. Assays for GUS activity indicate that the highest level of transient expression was in protoplasts isolated from 20-day old cells.

Keywords: Liriodendron tulipifera, electroporation, protoplasts, polyethylene glycol, transient expression, ß-glucuronidase

INTRODUCTION

Gene transfer technology can be applied to forest species for which regeneration systems have been developed. Yellow-poplar (Liriodendron tulipifera) is an ideal candidate for genetic transformation because it can be regenerated from cultured cells by somatic embryogenesis (Merkle and Sommer 1986). Protoplasts from embryogenic suspension cultures will form callus that retains developmental potential (Merkle and Sommer 1987). Methods involving the direct DNA transformation of protoplasts can be used as an alternative to Agrobacterium-mediated transformation. This approach avoids the host-range limitations of Agrobacterium.

Protoplasts can be induced to take up foreign DNA with a brief exposure to a high intensity electric field (electroporation) or polyethylene glycol (PEG). These techniques must be optimized for each species and tissue source. The efficiency of DNA uptake can be measured by the degree of transient expression of a reporter gene. In these studies, the gene encoding -glucuronidase (GUS) was introduced into yellow-poplar protoplasts and its expression was quantitated by a fluorometric assay (Jefferson et al. 1987).

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METHODS

Protoplast Preparation

Protoplasts were isolated from two embryogenic suspension culture lines by a modification of the procedures of Merkle and Sommer (1987). Briefly, approximately one gram of tissue was placed in 10 ml of filter-sterilized digestion medium containing 1% Cellulysin and 0.5% Macerase. After 24 hours of incubation at 30^0 C, the cells were filtered through Miracloth, washed twice by sedimentation, and sieved through a 25 pm pore stainless steel mesh. The length of time between subculture of the cell suspensions and protoplast preparation was varied. The average number of protoplasts/g after 5-day and 20-day intervals was 6.4×10^6 and 1.7×10^7, respectively.

Transformation of Protoplasts

The protoplast concentration was adjusted to 1×10^6 protoplasts/ml for electroporation and 2×10^6 protoplasts/ml for PEG-mediated transformation. Uncut plasmid DNA was added at a final concentration of 25 g/ml . Either pBI221, which carries the CaMV 35S/GUS/NOS construct, or pBI121, which carries the NOS/NPTII/NOS construct in addition to the same GUS construct, was used (Jefferson et al. 1987). A GUS construct with a soybean heat shock promoter was a gift from W. A. Ainley, Botany Dept., UGA. Protoplasts were electroporated with a Promega Model 450 in a buffer containing 100 mM NaCl, 4 mM $CaCl_2$, 8 % PEG, and 500 mM mannitol. The voltage, capacitance, and pulse length were varied. The pulse decay constant was calculated as the product of the capacitance and resistance (Fromm et al. 1987). Since the initial voltage (V_0), capacitance (C), and pulse length (t) are set and the voltage remaining after the pulse (V_t) is observed, the resistance (R) could be calculated from the following equation:

t

For. PEG-mediated transformation, the protoplast/DNA suspension was mixed with one volume of 40% PEG-6000 and incubated at room temperature for 45 minutes. PEG-treated and electroporated protoplasts were washed with yellow-poplar regeneration medium I (Merkle and Sommer 1987) and incubated for 18 hours at 300 C in a thin layer of the same medium. Controls without DNA were always included. Protoplast viability was determined by exclusion of 0.5% Evan's Blue dye. After transformation with pBI121, a fraction of the protoplasts was plated in agarose droplets (Merkle and Sommer 1987) and were selected with 100 µg/ml kanamycin after two weeks.

ß-Glucuronidase Assay

Protoplasts were sedimented at 100 x g in 15 ml Falcon tubes for 5 minutes and the pellet was resupended in 50 µl of GUS extraction medium (Jefferson et al. 1987). The suspension was transferred to eppendorf tubes, homogenized briefly with a glass pestle, and spun for 5 minutes in a microcentrifuge. The supernatant was removed and a 10 l aliquot was taken for protein quantitation by Bradford assay (Bio-Rad kit procedures). An equal volume of extraction buffer containing 2 mM 4-methyl umbelliferyl glucuronide was added to the protein extracts and incubated at 37^0 C. The reaction was stopped at different timepoints with the addition of 0.5 ml of 0.2 M Na_2CO_3. ß-Glucuronidase

hydrolyzes the substrate, producing methyl umbelliferone (MU), which is fluorescent when excited by long-wave UV light. The fluorescence of the samples was quantitated with a Hoefer TKO 100 Mini-Fluorometer and the MU concentration was determined from a standard curve. Background fluorescence, determined from the controls without DNA additions, was subtracted and GUS activity was normalized on a per mg protein basis.

RESULTS AND DISCUSSION

Electroporation Parameters

Sucessful transformation by electroporation requires a degree of membrane permeablization that balances gene transfer efficiency with cell viability. Generally, this involves electroporation conditions that have a decay time constant of 5-15 ms and lead to a reduction of 50% in protoplast viability (Fromm et al. 1987). The decay time constant is a function of the capacitance of the electroporator, the voltage to which it is charged, and the resistance of the buffer containing the protoplasts.

Figure 1 shows the effect of field strength (V/cm) on protoplast viability. With a capacitance of 450 microfarads (pF), cell viability was reduced to 24% when a field strength of 800 V/cm was applied. A capacitance of 450 pF was used in further experiments because the higher capacitance did not reduce viability significantly. With a field strength of 700 V/cm, protoplast viability was 53%, which is nearly optimal. The resistance of the electroporation buffer, determined by its ionic strength, was held constant. When the pulse length was set to 30 ms, and with a capacitance of 450 pF and a field strength of 700 V/cm, the decay time constant was calculated to be 12 ms.

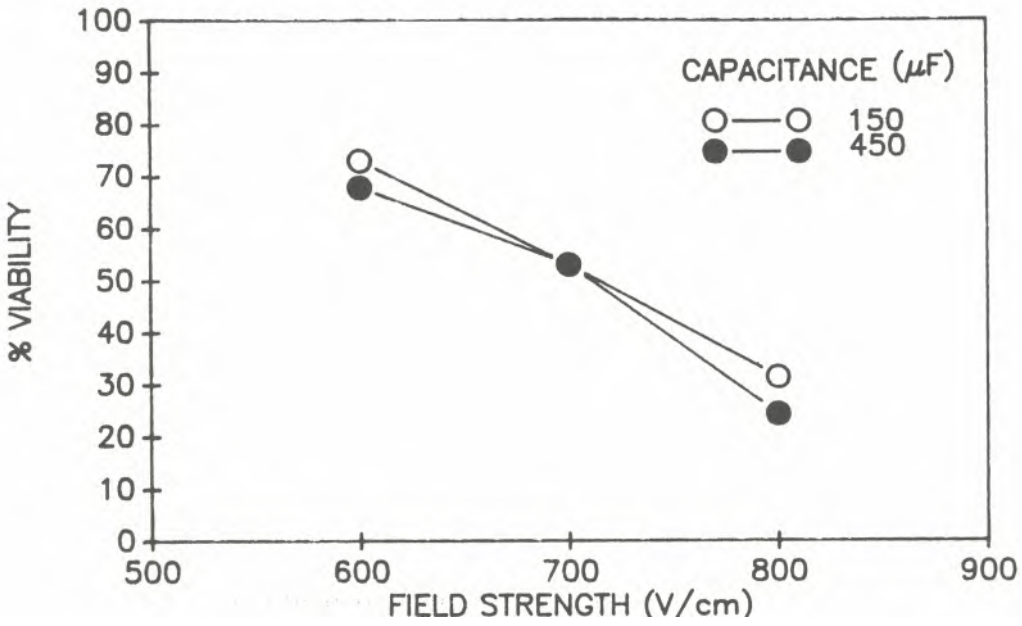

Figure 1. Effect of field strength on protoplast viability. Protoplasts were electroporated and viability was assessed the following day by dye exclusion.

At a field strength of 600 V/cm, protoplast viability remained near 70%. It is typical for small, embryogenic cells to require higher field strengths for membrane permeabilization than larger, vacuolated cells from, for example, mesophyll tissue. Yellow-poplar protoplasts isolated from embryogenic cell suspensions are small (10-20 pm diameter) and densely cytoplasmic. Electroporation conditions for these protoplasts are comparable to those for other protoplasts with regenerative potential. Table 1 shows electroporation parameters for yellow-poplar, rice (Toriyama et al. 1988), carrot (Fromm et al. 1985), and maize (Fromm et al. 1986).

Table 1. Electroporation parameters of protoplasts from suspension cultures with regenerative potential.

	Field strength	Capacitance	Decay constant
yellow-poplar	700 V/cm	450 pF	12 ms
rice	750	22	4
carrot	875	980	20
maize	500	245	4

Influence of culture cycle on transient expression

Yellow-poplar suspension cultures were subcultured into fresh medium every two weeks. Protoplasts were routinely isolated one week after subculture, but GUS expression was never detected after these protoplasts were electroporated with either pBI121 or pBI221. GUS was expressed transiently, however, in protoplasts isolated two and three weeks after subculture. To examine this variation in more detail, protoplasts were isolated at five-day intervals after a subculture and were incubated with pBI221 and 20% PEG. After washing and overnight incubation, the protoplasts were lysed and assayed for GUS activity. The results are shown in Figure 2.

PEG-mediated transformation of protoplasts isolated from culture line TP 14x108 at 5, 10, and 25 days after subculture produced low levels of GUS activity. The identical treatment of protoplasts isolated 15 and 20 days after subculture led to a 2-fold and 6-fold increase, respectively, in GUS activity. A similar, though less well pronounced, pattern of GUS expression was evident in protoplasts from tissue culture line TP 4x12. This suggests that the physiological age of the protoplasts may affect their ability to take up or express foreign DNA. The differential expression of the chaemeric gene does not appear to be related to the CaMV 35S promoter, because the same results were observed when a soybean heat shock promoter-GUS construct was used. Transformation efficiency was found to be affected by the mitotic state of the protoplasts in tobacco (Meyer et al. 1985, Okada et al. 1986). Yellow-poplar suspension cultures are asynchronous, however, so the contribution of a single mitotic state would be minimal.

When the PEG concentration was increased from 8 to 20%, PEG-mediated DNA uptake lead to levels of GUS activity in the range previously obtained by electroporation. This method of transformation was less deleterious to

protoplasts, as evidenced by improved colony production when these protoplasts were plated **in agarose** droplets. Kanamycin (100 µg/m1) was used to select colonies derived from protoplasts after PEG-mediated uptake of pBI121. This plasmid carries **a gene** encoding neomycin phosphotransferase II, which confers **antibiotic resistance. While** cells not exposed to pBI121 died, some colonies which had been treated with the plasmid survived. Similarly treated protoplasts from the same experiment showed transient GUS activity. Transformation of these colonies cannot be confirmed until DNA analysis has been completed.

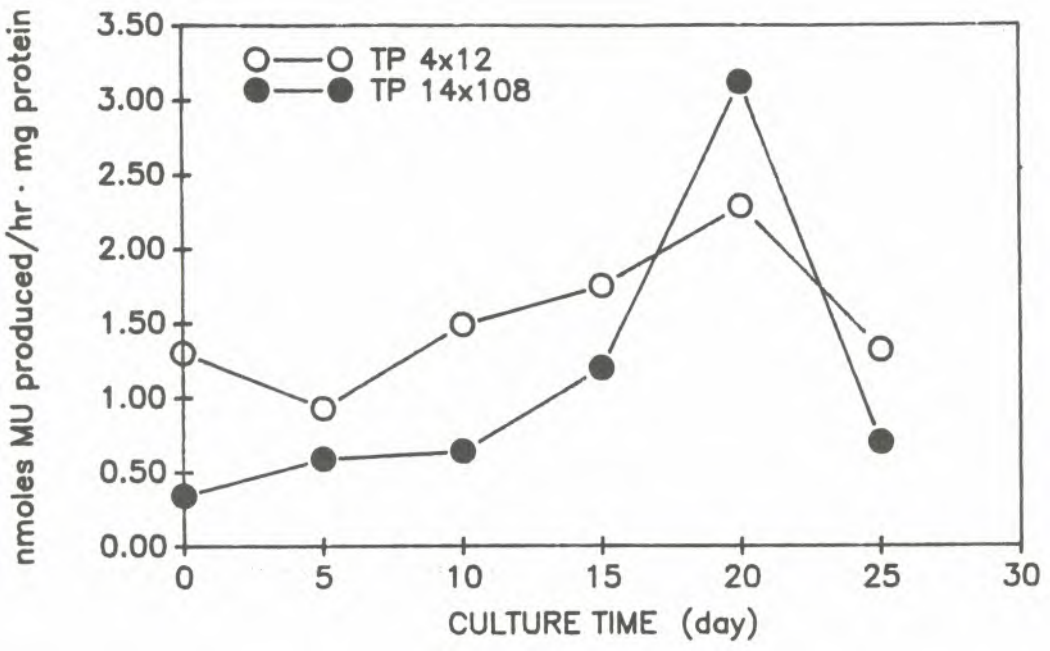

Figure 2. Transient GUS expression in protoplasts isolated at different timepoints after subculture. GUS activity shown is the average of two transformations per timepoint per experiment. The experiment was repeated twice for each culture line.

CONCLUSIONS

Plasmid DNA was introduced into yellow-poplar protoplasts by both electroporation and PEG-mediated uptake. Electroporation parameters were adjusted to give a pulse decay constant of 12 ms and protoplast viability of 53%. Conditions for electroporation of yellow-poplar protoplasts were in the range of those for small, developmentally active protoplasts from other species. Transient expression of the GUS gene showed that DNA uptake by PEG treatment alone was as effective as electroporation when the PEG concentration was raised to 20%. The physiological age of the protoplasts influenced the amount of GUS activity observed after the uptake of pBI221. Whether protoplasts at various stages of the culture cycle differ in the ability to take up or express the DNA is unknown. The introduction of an antibiotic gene from pBI121 has apparently permitted some colonies to survive on 100 µg/ml kanamycin.

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