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Transient gene expression in electroporated *Picea glauca* protoplasts*

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ABSTRACT

The reporter gene for chloramphenicol acetyltransferase (CAT) was introduced into white spruce (Picea glauca (Moench) Voss.) protoplasts by electroporation. CAT transient gene expression was increased by increasing the concentration of pCaMVCN plasmid and was affected by the level of the applied voltage. Highest CAT activities were obtained after electroporation with a pulse of 350V.cm^{-1} having an exponential decay constant of approximately 105ms. Linearized plasmid constructs gave much higher levels of CAT activity than circular plasmid. Attempts to use the Escherichia coli β-glucuronidase gene (β-GUS) as a marker gene revealed very high levels of β-GUS-like activity in electroporated protoplasts. This activity was mainly due to a small molecule and may mask successful transformation since β-GUS-like activity increased when plasmid DNA was present during electroporation.

ABBREVIATIONS

CAT: chloramphenicol acetyltransferase, β-GUS: β-glucuronidase, MUG: 4-methyl umbelliferyl glucuronide, μF: microfarads.

INTRODUCTION

White spruce is an important forest tree in Canada and the USA and has been the subject of many recent studies in vitro aimed at tree improvement and production programmes e.g., somatic embryogenesis (Hakman and Fowke, 1987), maturation of somatic embryos into plantlets (Hakman and von Arnold, 1988, Dunstan et al., 1988) cryopreservation (Kartha et al., 1988), and protoplast culture and regeneration of proembryos (Bekkaoui et al., 1987; Attree et al., 1987). These recent developments lead naturally to investigations concerning genetic engineering of white spruce protoplasts that could allow us to make specific genetic changes in a short period of time, as compared to traditional plant breeding methods. For example, it may be profitable to create transgenic white spruce plants carrying the Bacillus thuringiensis toxin gene or other genes useful for agronomic improvement.

Electroporation stimulates uptake of DNA into plant protoplasts permitting transient expression of foreign genes and production of stable transformants in several species (Fromm et al., 1985; Shillito et al., 1985; Hauptman et al., 1987; see Morikawa et al., 1988 for review). Electroporation has the potential to be a high efficiency method of DNA delivery (Potrykus et al., 1987) because it does not have the host range limitations of virus or Agrobacterium-mediated gene transfer methods; it is simple and permits rapid evaluation of the functionality of gene constructions.

In this report we characterize factors influencing the transient expression of the chloramphenicol acetyltransferase (CAT) gene introduced by electroporation into protoplasts obtained from embryogenic cell suspensions of white spruce. Attempts were also made to use the Escherichia coli β-glucuronidase gene (β-GUS) as a reporter gene.

MATERIALS AND METHODS

Protoplast Isolation and Culture

Protoplasts from 5 to 6-day-old subcultures of embryogenic suspension cultures were isolated as previously described (Bekkaoui et al., 1987; Attree et al., 1987). Briefly, 2.5-3.0g fresh weight of cells were incubated with 10 ml of enzyme solution containing 0.5% (w/v) Cellulase R-10, 0.25% (w/v) each of Pectinase and Driselase, 5mM $CaCl_2.2H_2O$ and 0.5M mannitol. After purification on a sucrose-mannitol gradient, the protoplasts were resuspended in culture medium. Viability was measured by the exclusion of phenosafranine (Widholm, 1972), at least 500 protoplasts per treatment were counted. Yields were typically 0.8-1.0 x 10^6 protoplasts per g of fresh weight with viabilities of 75-85%.

Electroporated, and non-electroporated protoplasts were cultured in liquid LP medium with 1% sucrose (von Arnold and Eriksson, 1981) supplemented with 0.38M glucose, 5mM glutamine, 2mg.L^{-1} 2,4D-dichlorophenoxyacetic acid and 1mg.L^{-1} benzylaminopurine, at a density of 2 to 5 x 10^4 protoplasts per ml, 40ml per Petri dish (9 cm diameter). Protoplasts were cultured 48 hours in moistened plastic boxes under diffuse light (7-10 μE m^{-2} s^{-1}) at 25 ± 1°C before being collected for enzymatic assays.

Electroporation

A 0.8ml aliquot, containing 1 x 10^6 protoplasts in LP medium supplemented with 0.38M glucose was placed in a Bio-Rad 1 ml electroporation cuvette with a 0.4cm space between the electrodes. Sonicated calf thymus

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DNA was added as carrier DNA at a concentration of 50 $\mu g.ml^{-1}$. After addition of plasmid DNA at various concentrations (0, 20, 50, 100 $\mu g.ml^{-1}$), the protoplasts were kept on ice for 10 min. The sample was then subjected to electroporation at 960 µF and various voltages with a Bio-Rad Gene Pulser apparatus, which generated an exponential decay pulse of time constant varying between 100 and 120ms. The time constant of the pulse is the decay time from peak voltage to 37% of peak voltage. After electroporation, the samples were kept on ice for 10 min. before being diluted and cultured.

Plasmids

The plasmid pBI221 was obtained from Clonetech Laboratories Inc. (Palo Alto., Ca, USA). This 5.7Kb vector contains the 3.0Kb HindIII EcoRI fragment from pBI121 (Jefferson et al., 1987) which carries the cauliflower mosaic virus (CaMV) 35S promoter, the β-GUS gene from E. coli and the NOS poly A site from the Agrobacterium tumefaciens Ti plasmid. The plasmid pCaMVCN 4.2 Kb carrying the CaMV 35S promoter, the CAT gene and the NOS poly A site was obtained from Pharmacia. Both plasmids were maintained in E. coli DH5-α strains and prepared by CsCl gradient centrifugation. Linearization of pCaMVCN was accomplished by restriction digestion of the ClaI site.

CAT Assay

The method of Gorman et al. (1982) was used to determine CAT activity. Briefly, protoplasts (2 x 10^6) were collected by centrifugation and the pellets resuspended in 500µl of buffer (0.225M Tricine, pH 7.8, 0.5mM phenylmethyl sulphonyl fluoride and 5mM EDTA) prior to thorough homogenization in a glass homogenizer. The extracts were clarified by centrifugation in a microcentrifuge for 10min., and the supernatant was then transferred to a fresh tube and heated 10min. at 65°C. Acetyl-CoA (final concentration 1mM) and ^{14}C-chloramphenicol (1µCi, $60mCi.mmol^{-1}$, NEN, Dupont) were added and the reaction was allowed to proceed for 1 hour at room temperature before being terminated by the addition of 10 volumes of ethyl acetate. As a control 0.5 unit of commercial CAT was used (Pharmacia). After vortexing and clarification by centrifugation, the supernatant was transferred to a fresh tube, and dried under nitrogen in a hot water bath, redissolved in 30µl of ethyl acetate and submitted to ascending chromatography in chloroform/methanol (95:5 v/v) on silica gel plates. Separated spots of ^{14}C-chloramphenicol and its acetylated forms were visualized by autoradiography on X-OMAT Kodak film for 3-4 days and ^{14}C-acetylated chloramphenicol spots were quantified by liquid scintillation counting.

β-GUS Assay

Protoplasts, collected by centrifugation and frozen in liquid nitrogen were homogenized by grinding in a lysis buffer (Jefferson et al., 1987) consisting of 50mM sodium phosphate (pH7.0), 10mM β-mercaptoethanol, 0.1% (w/v) Triton X-100 and 0.1%(w/v) Sarkosyl. Extracts or fractions of 200µl were assayed in the lysis buffer containing 1mM MUG substrate (Sigma) made up to a final volume of 500 µl. The reaction was allowed to proceed at 37°C for 15 hours and terminated by adding 4.5 ml of 0.2M Na_2CO_3, fluorescence emission was measured using a fluorescence spectrophotometer (Turner, model 111) under an excitation wavelength of 365 nm and an emission filter of 455 nm. The fluorimeter was calibrated by making standard curves with methylumbelliferone (MU) solutions of known concentrations.

Protein content in extracts or fractions was determined using the method of Bradford (1976) (Bio-Rad Protein Assay kit); bovine serum albumin was used as standard. When fractionation was performed, 0.5ml extracts from 2 x 10^6 protoplasts were passed through a Sephacryl S-300 column superfine 0.5 x 16 cm (Sigma) at 4°C, using the lysis buffer without detergents as elution buffer. The flow rate was $0.1ml.min^{-1}$ with fractions of 250-300 µl. Commercial β-GUS (Sigma) was used as a control in fractionation experiments. All the experiments were repeated at least twice. Variabily of the two replicates was less than 50%.

RESULTS

CAT Marker Gene

Using the pCaMVCN plasmid in electroporation experiments (960 µF, $250V.cm^{-1}$) resulted in detection of transient expression of the CAT gene. The voltage of electroporation at 960 µF was an important factor affecting subsequent levels of CAT activity and viability (Fig. 1). Increasing the voltage between $250V.cm^{-1}$ and $400V.cm^{-1}$ resulted in viability decreasing from 49% to 10%, while the detected CAT activity increased significantly. Above this voltage ($500V.cm^{-1}$ or more), viability fell below 5% and a decline in transient CAT activity was observed (data not shown). The capacitance was also an important factor in obtaining transient CAT activity. For example 3 µF, $750V.cm^{-1}$, (11% viability) did not lead to significant levels of transient expression.

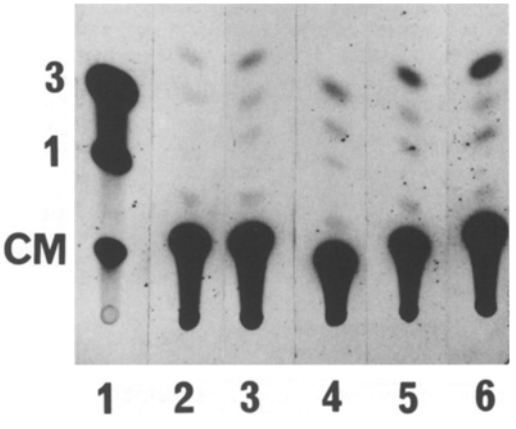

Fig. 1. Effect of voltage on CAT activity. White spruce protoplasts were electroporated at 960µF in the presence of $50\mu g.ml^{-1}$ pCaMVCN and CAT assays performed as described in "materials and methods". The autoradiogram of a TLC plate shows: 1-^{14}C-acetyl chloramphenicol (1), 3-^{14}C-acetyl chloramphenicol (3) and ^{14}C-chloramphenicol (CM), Lane 1: CAT assay with commercial enzyme; voltage applied and viability, 2: $0V.cm^{-1}$ and 73%, 3: $250V.cm^{-1}$ and 49%, 4: $300V.cm^{-1}$ and 29%, 5: $350V.cm^{-1}$ and 17%, 6: $400V.cm^{-1}$ and 10%.

The concentration and shape of the pCaMVCN plasmid used during electroporation were also important factors influencing transient CAT activity (Fig. 2). Plasmid concentrations of 20, 50 and 100 $\mu g.ml^{-1}$, at $300V.cm^{-1}$ resulted in increasingly higher levels of CAT activity with increasing DNA concentration, i.e. 2, 5 and 10 times more, respectively, than control (without plasmid). Furthermore, under the same

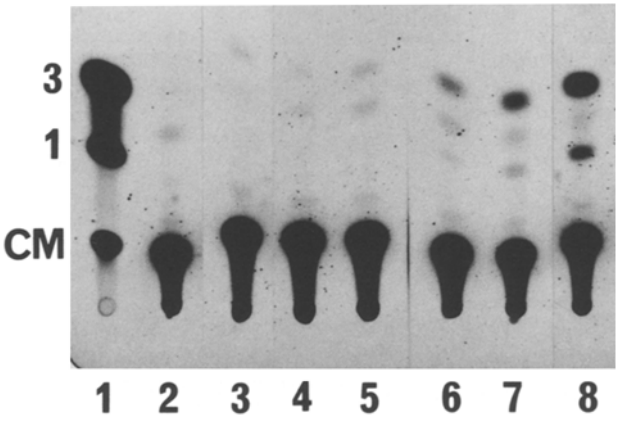

Fig. 2. Effect of pCaMVCN concentration and circular or linear form of the plasmid on CAT activity. White spruce protoplasts were electroporated at 960µF, 300V.cm^{-1} and CAT assays were performed as described in "materials and methods". The autoradiogram of a TLC plate shows: 1-^{14}C-acetyl chloramphenicol (1), 3-^{14}C-acetyl chloramphenicol (3) and ^{14}C-chloramphenicol (CM). Lane 1: CAT assay with commercial enzyme, Lane 2: Control without protoplast extract showing background due to commercial reaction components, Lane 3: Protoplasts without electroporation, Lane 4: Electroporation without DNA, Lane 5: Electroporation with 20µg.ml^{-1} DNA, Lane 6: Electroporation with 50µg.ml^{-1} DNA, Lane 7: Electroporation with 100 µg.ml^{-1} DNA, Lane 8: Electroporation with 100 µg.ml^{-1} linear DNA.

conditions, linearized pCaMVCN DNA gave 5 to 10 times higher levels of CAT activity than circular DNA.

β-GUS Marker Gene

First attempts to develop transient β-GUS expression resulted in higher β-GUS activity in protoplasts electroporated (960 µF, 250 V.cm^{-1}) in the presence of pBI 221 plasmid than in the control. However, it was found that much higher levels of β-GUS-like activity could also result following electroporation without plasmid, under conditions which resulted in low (<1%) viability (960 µF, 1000 V.cm^{-1}). In an effort to determine the nature of the background activity, extracts from protoplasts electroporated without plasmid DNA (960µF, 250V.cm^{-1}, viability 53%) were fractionated, and protein content and β-GUS-like activity were measured in each fraction. It was found (Fig. 3A) that some of the β-GUS-like activity (<20% of total activity) fractionated with the proteins, peaking in fractions 11 or 12 (peak a), while the bulk of the background activity (>80% of total) peaked in fraction 23(peak b), where no proteins were detectable using the Bio-Rad macroassay. When protoplasts were electroporated in the presence of 50µg.ml^{-1} pBI 221 plasmid DNA (viability 36%), both of the peaks were found to increase (Fig. 3B). However, after protoplasts were electroporated at 1000V.cm^{-1}, the total protein content and peak a decreased, while peak b increased to very high levels (Fig. 3C). All the reported β-GUS-like activities

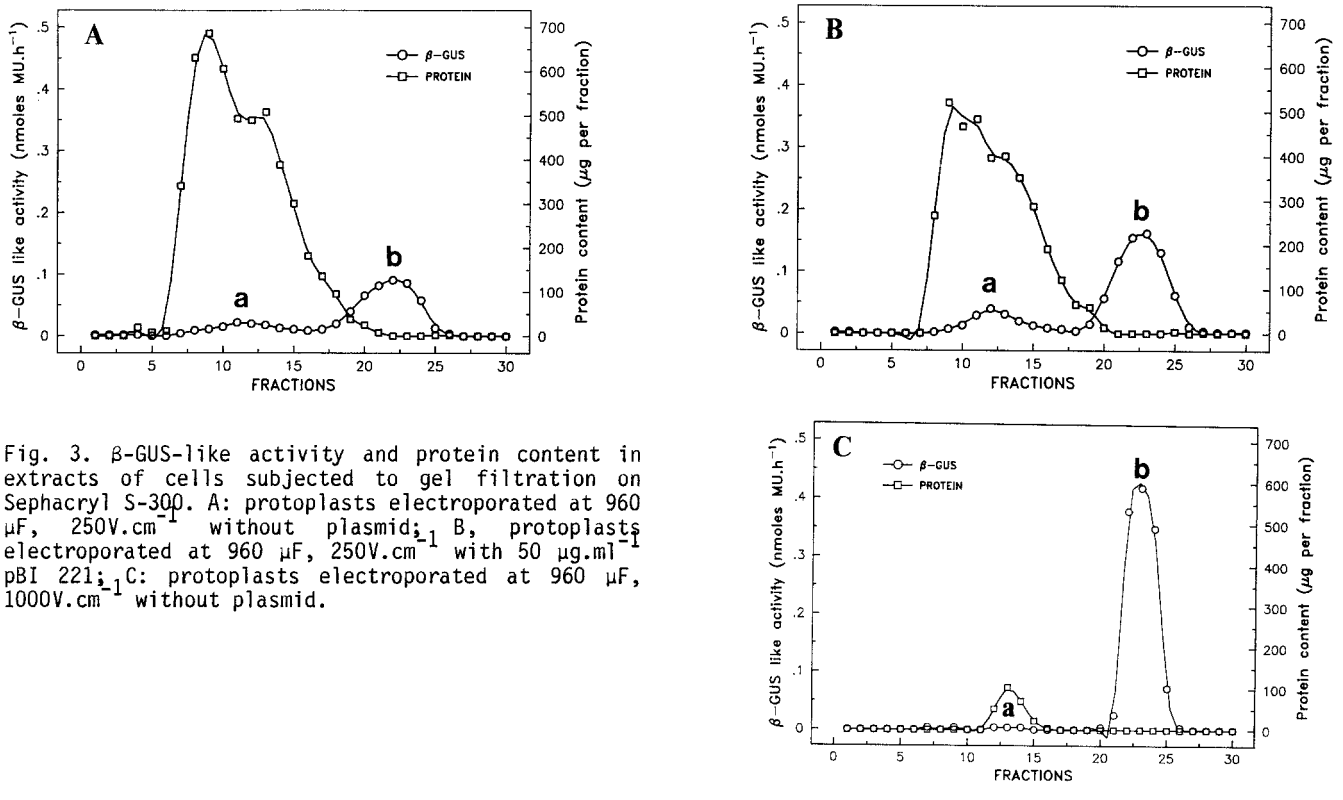

Fig. 3. β-GUS-like activity and protein content in extracts of cells subjected to gel filtration on Sephacryl S-300. A: protoplasts electroporated at 960 µF, 250V.cm^{-1} without plasmid; B, protoplasts electroporated at 960 µF, 250V.cm^{-1} with 50 µg.ml^{-1} pBI 221; C: protoplasts electroporated at 960 µF, 1000V.cm^{-1} without plasmid.

were found to be MUG dependent. The commercial β-GUS enzyme (Sigma) was found to have a fractionation pattern similar to peak a. The background levels observed when using the substrate MUG to detect β-GUS activity reduced the usefulness of β-GUS as a reporter gene as compared to the CAT gene.

DISCUSSION

Transient expression of the CAT gene clearly demonstrates that electroporation can be used to introduce foreign genes into white spruce protoplasts. Although $400V.cm^{-1}$ gave the highest levels of transient expression, the viability was only 10%. For this reason we feel that using lower voltages such as $350V.cm^{-1}$ or $300V.cm^{-1}$ may be preferable for regeneration of stable transformed somatic embryos. The levels of CAT transient expression obtained were proportional to the initial concentration of pCaMVCN plasmid as previously reported in non-coniferous plants (Fromm et al., 1985; Prols et al., 1988). Similarily, using linearized plasmid DNA compared with supercoiled DNA resulted in higher levels of transient expression, which is in agreement with results obtained with non-coniferous plants (Shillito et al., 1985) and animal cells (Potter et al., 1984).

The results with the β-GUS marker gene suggest that the background β-GUS-like activity observed in electroporated white spruce protoplasts may come from two different sources. The increase in peak a, following electroporation in the presence of pBI 221 plasmid may reflect transient expression of the β-GUS marker gene. Peak b may be due to compounds released by damaged protoplasts following electroporation. Although the β-GUS marker gene has been used successfully with other species to monitor transient expression following electroporation (Seguin and Lalonde, 1988; Topfer et al., 1988), our results indicate that electroporated white spruce protoplasts release a small molecule that mimics β-GUS activity in the fluorescent assay. Jefferson (1987) observed that endogenous compounds and enzymes released after lysis can cause fluorescence, although he noted that this fluorescence should not be MUG dependent. The fluorescence observed in our experiments was MUG dependent.

Our results show that it is possible to induce DNA uptake and transient gene expression in white spruce protoplasts using electroporation. Our results also suggest that CAT may be a preferable reporter gene to β-GUS under our conditions. Experiments leading to stable transformation and plantlet regeneration are in progress.

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