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Factors affecting transient gene expression in electroporated black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) protoplasts *

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Summary. Methods were developed for transient gene expression in protoplasts of black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*). Protoplasts were isolated from embryogenic suspension cultures of black spruce and from non-embryogenic suspensions of jack pine. Using electroporation, transient expression of the chloramphenicol acetyltransferase (CAT) gene was assayed and shown to be affected by the cell line used, by voltage, temperature, and by the plasmid concentration and conformation. Increasing the plasmid DNA concentration (0–150 μg ml^{-1}) resulted in higher levels of transient CAT expression. In jack pine, linearized plasmid gave 2.5 times higher levels of CAT enzyme activity than circular. Optimal voltage varied for each cell line of the two species within the range 200–350 V cm^{-1} (960 μF). A heat shock treatment of protoplasts for 5 min at 45 °C resulted in enhanced CAT gene expression for both species.

Key words: Conifers – Protoplasts – Electroporation – Chloramphenicol acetyltransferase – Temperature

Introduction

Black spruce (*Picea mariana* Mill.) and jack pine (*Pinus banksiana* Lamb.) dominate much of the Canadian boreal forest and are valuable resources to the forest industry (Anon 1986; Attree et al. 1989). Due to a combination of factors, however, including the occurrence of fires, insect predation, and insufficient resource management, there is an urgent need for large numbers of genetically improved trees with the capacity for rapid growth and shortened rotations (Thorpe and Biondi 1984; Dunstan 1988).

One avenue to genetic improvement is the use of direct gene transformation using, e.g., electroporation (Morikawa et al. 1988; Shigekawa and Dower 1988). Further, because electroporation does not suffer from the host-range limitations of viral or *Agrobacterium*-mediated transfer methods, it is useful in the rapid evaluation of the functionality of plasmid constructions (Okada et al. 1986; Werr and Lörz 1986; Ebert et al. 1987; Ballas et al. 1988) and for assessing transient (Fromm et al. 1985; Bates et al. 1988) and stable transformation (Langridge et al. 1985; Shillito et al. 1985; Fromm et al. 1986).

Recently, transient chloramphenicol acetyltransferase (CAT) gene expression in conifer protoplasts was obtained in white spruce, using electroporation (Bekkaoui et al. 1988) and polyethylene glycol (PEG)-mediated DNA uptake (Wilson et al. 1989). Both reports describe the use of 35S cauliflower mosaic virus (CaMV) promoter-driven CAT, and compare the effects on transient expression of linear versus circular plasmid. In each case single, but different, cell lines of white spruce were used. Transient expression of the luciferase gene, driven by a 35S promoter, was also obtained following electroporation of protoplasts from single cell lines of Douglas fir and loblolly pine (Gupta et al. 1988).

The purpose of this study was to determine if electroporation was applicable for use with black spruce and jack pine, to document the degree of within-species variation, and to determine to what extent it was possible to optimize transient CAT expression. This was accomplished by variation of electroporation parameters, assessments of relative promoter strengths, and by deter-

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mining the effects of use of carrier DNA and linear versus circular plasmid. In addition, the effects of cold and heat-shock temperatures were investigated.

Materials and methods

Origin and description of suspension cultures

Seeds of *Picea mariana* (Mill.) were collected from the Hudson Bay area, Saskatchewan, in 1975. Seeds of *Pinus banksiana* (Lamb.) were collected from the Smeaton area, Saskatchewan, in 1980. All seeds were stored at −18 °C until use. Seeds were surface-sterilized for 12 min by agitation using 15% commercial bleach (Javex, 6.0% sodium hypochlorite), to which was added several drops of Tween 80, followed by 2 min immersion in 70% ethanol, and 5 rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from seeds and inoculated onto 1/2 strength modified Litvay medium (LM) (Litvay et al. 1981) containing 60 m*M* sucrose, 0.1 m*M* $FeSO_4 \cdot 7\,H_2O$, 0.1 m*M* Na_2EDTA, 3.0 m*M* glutamine, 1.0 g l^{-1} casein hydrolysate, 9 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma Chemical), and 4.5 μ*M* N^6-benzylaminopurine (BA) (Sigma), solidified with 8.0 g l^{-1} agar (pH 5.8). Cultures were incubated at 25° ± 2 °C in the dark.

In black spruce, white, mucilaginous, embryogenic callus was isolated from zygotic embryos (approximately 10% frequency), after 2–3 months incubation. This callus is similar in appearance to that reported by Hakman and Fowke (1987), who established embryogenic calli of black spruce from immature zygotic embryos. Explants of jack pine produced green non-embryogenic callus after 1–2 months incubation. The callus described were used in the production of embryogenic cell suspensions of black spruce (four cell lines: BSA, BSD, BSF, BSG) and non-embryogenic suspensions of jack pine (three cell lines: JP1, JP2, JP3). Each cell suspension line was initiated from a different genotype. Suspensions were maintained in 50 ml (250-ml Delong flasks) of 1/2 strength modified LM (as described above) and agitated on a gyratory shaker (150 rpm) under continuous irradiance of 15–20 μE $m^{-2}s^{-1}$ at 25° ± 2 °C. Subcultures were made at weekly intervals (20% v/v). The cell suspensions used in this study had been maintained for at least 3 months prior to commencing these experiments.

Protoplast isolation and culture

Black spruce and jack pine protoplasts were isolated following the methods of Bekkaoui et al. (1987). Cells from an actively growing suspension culture were collected on Miracloth (Chicopee Mill/NY) and transferred to petri dishes (100 × 25 mm; Nunc/IL) containing a cell wall digesting solution (10 ml per 2 g fresh weight of cell suspension). The enzyme solution consisted of 1.0% (w/v) Cellulase (Onozuka R10, Yakult Honsha, Tokyo), 0.25% each of Hemicellulase (Sigma), Driselase (Sigma), Pectinase (Sigma), 5 m*M* $CaCl_2 \cdot 2\,H_2O$, and 0.5 *M* mannitol (pH 5.8).

Cells were incubated in enzyme solution for 5–7 h on a gyratory shaker (50 rpm) at room temperature in the dark. After purification on a sucrose-mannitol gradient, the protoplasts were resuspended in 1/2 strength modified LM (as described above) supplemented with 0.37 *M* glucose. Final osmolality of the protoplast culture medium was 520 mOsm kg H_2O^{-1} as measured by a micro-osmometer (Precision Instruments, Natick/MA). Protoplast viability was measured by both visual observation and the exclusion of phenosafranine (Widholm 1972) immediately after isolation and at the time of CAT assay. The viability of electroporated protoplasts are given as relative viabilities for direct comparison within treatments. At least 500 protoplasts were counted for each treatment. Viabilities immediately after purification were 75%–85% for both species. Yields of protoplasts were typically $2-3 \times 10^5$ and $3-4 \times 10^5$ per gram of fresh weight from black spruce and jack pine suspensions, respectively, as determined by haemocytometer counts.

Following electroporation, protoplasts were plated at a density of 5×10^4 protoplasts per ml, 20 ml per petri dish (100 × 25 mm). All cultures were sealed with Parafilm (American Can), maintained at 25° ± 2 °C in moist plastic boxes under diffuse light (7–10 μE $m^{-2}s^{-1}$; 16 h photoperiod), and incubated for 24 h before being collected for enzymatic assays.

Electroporation

Each 0.8 ml aliquot of suspension containing 1×10^6 protoplasts was placed in a Bio-Rad (Richmond/CA) 1-ml capacity electroporation cuvette with a 0.4-cm space between the electrodes. Sonicated calf thymus DNA was added as a carrier DNA at various concentrations (0–150 μg ml^{-1}). After addition of plasmid DNA at various concentrations (0–150 μg ml^{-1}), protoplast samples were kept on ice for 10 min before being subjected to electroporation at 960 μF and various voltages with a Bio-Rad Gene Pulser transfection apparatus. The apparatus generated an exponential decay pulse of time constant (i.e., the decay time from peak voltage to 37% of peak voltage) varying between 160–220 ms (with ice incubation before and after electroporation) for both species (200, 250, 300 V cm^{-1}). At voltages higher than 300 V cm^{-1}, the time constant varied from 130 to 160 ms. In the absence of an ice incubation prior to electroporation, the time constant varied from 90 to 100 ms for both species. After electroporation, samples were routinely kept on ice for 10 min before being plated.

In order to determine the effect of temperature on CAT activity, protoplasts were incubated after the addition of DNA in one of four ways: (1) 10 min on ice before electroporation; (2) 10 min on ice after electroporation; (3) no ice incubation; (4) a combination of (1) and (2) above.

In some experiments, protoplasts were heat-shocked at 45 °C for 5 min before addition of plasmid DNA, followed by incubation on ice before and after electroporation, or no ice incubation.

Plasmids

The plasmids pCaMVCN and pNCN were obtained from Pharmacia (USA). The pCaMVCN plasmid is a 4.2-kb vector which contains the cauliflower mosaic virus (CaMV) 35S promoter, the CAT gene, and the nopaline synthase (NOS) terminator. The pNCN is a 5.2-kb vector which contains the NOS promoter, the CAT gene, and the NOS terminator. Both plasmids were maintained in the *E. coli* strain DH5-α and prepared by CsCl gradient centrifugation. Plasmids were sterilized by ethanol precipitation and dissolved in 1/2 strength modified LM medium at 1 mg ml^{-1}. Linearization of pNCN was accomplished by restriction digestion of the unique XbaI site.

CAT assay

The method of Gorman et al. (1982) as modified by Fromm et al. (1985) was used to determine CAT activity with the following changes: protoplasts were used at a density of 1×10^6, and only 0.2 μCi [^{14}C]-chloramphenicol (CM) (60 mCi $mmol^{-1}$, NEN, Dupont) was added to protoplast extracts. As a control, 0.25 units of commercial CAT was used (Pharmacia).

Separated spots of chloramphenicol and its acetylated forms were visualized by autoradiography on X-OMAT Kodak film for 3–4 days, and radioactive spots were quantified by liquid scintillation counting (LKB Wallac 1217 Rackbeta). The percentage of CM which was acetylated (AcCM) was calculated as

Table 1. Effect of voltage and cell line on CAT activity and relative protoplast viability

Cell line	Voltage (V cm^{-1})	% Ac CM[a]	% Protoplast viability
Black spruce			
BSA	0	N.D.[b]	
	200	0.1	23
	250	0.1	14
	300	0.1	10
BSD	0	N.D.	
	200	0.8	14
	250	1.5	7
	300	0.5	5
BSF	0	0.2	63
	200	7.0	50
	250	35.0	45
	300	20.0	30
BSG	0	N.D.	
	200	0.3	18
	250	0.3	10
	300	0.3	7
Jack pine			
JP1	0	0.1	71
	200	1.5	52
	250	2.3	43
	300	3.2	24
	350	1.3	8
JP2	0	0.1	67
	200	0.7	57
	250	1.6	35
	300	3.6	30
	350	1.1	9
JP3	0	0.1	71
	200	0.6	61
	250	1.6	26
	300	0.6	4
	350	0.8	3

[a] % Ac CM is the cpm CM acetylated/cpm total CM (per 10^6 protoplasts). Plasmid (pCaMVCN) was added at 100 μg ml^{-1} and carrier DNA at 50 μg ml^{-1} in all treatments
[b] N.D. – not determined

cpm Ac CM/cpm total CM. Unless otherwise indicated, all values are a mean of at least two independent experiments. Variability between experiments was less than 30%.

Results

Effect of voltage and cell line

Initial experiments were focused on determining the best cell line and voltage for transient CAT gene expression in each species. In black spruce, the cell line BSF was shown to exhibit maximal CAT activity with 35% Ac CM at 250 V cm^{-1} (45% viability) (Table 1). Viability decreased in black spruce (BSF) from 63% to 30% over the 0–300 V cm^{-1} range. In jack pine, the maximum CAT activity of 3.6% Ac CM was expressed in the cell line JP2 at 300 V cm^{-1} (30% viability) (Table 1). Viability decreased in jack pine (JP2) from 67% to 9% over the 0–350 V cm^{-1} range. In jack pine, optimal voltage for CAT gene expression in the cell line JP3 was shown to be 250 V cm^{-1} and not 300 V cm^{-1}, as seen with lines JP1 and JP2. In all further experiments, the cell lines BSF and JP2 were used at 250 and 300 V cm^{-1}, respectively. The addition of plasmid to protoplasts without an electric pulse, or an electric pulse applied to protoplasts without the addition of plasmid resulted in less than 0.5% CM acetylation in both species.

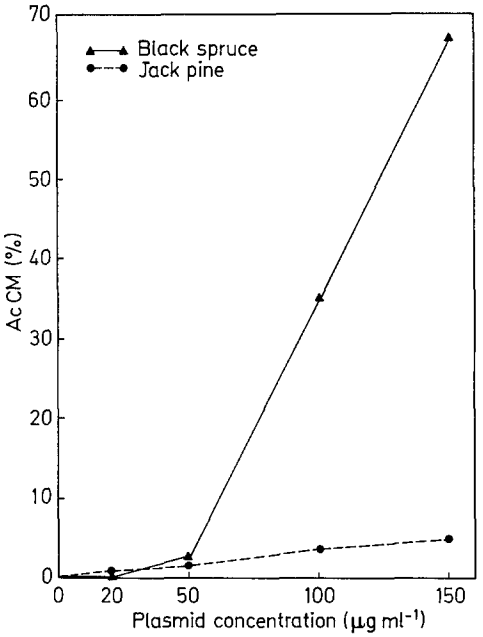

Fig 1. Effect of pCaMVCN concentration on CAT activity in electroporated black spruce and jack pine protoplasts. Carrier DNA was used at 50 μg ml^{-1} in all treatments

Plasmid concentration and presence of carrier DNA

The concentration of the pCaMVCN plasmid used during electroporation was an important factor influencing transient CAT activity in both species. As seen in Fig. 1, jack pine protoplasts showed an increase in CAT activity with increased DNA concentration, although activity was only 5%, even at plasmid concentrations as high as 150 μg ml^{-1}. In black spruce, a substantial increase in CAT activity occurred at concentrations above 50 μg ml^{-1} pCaMVCN. At 150 μg ml^{-1} of plasmid, 68% Ac CM was observed in this species. Only trace activity was observed in black spruce at concentrations below 50 μg ml^{-1}. For convenience, in all other experiments plasmid DNA was used at 100 μg ml^{-1}.

Since CAT activity was low in jack pine, experiments were conducted to determine the effect of adding various

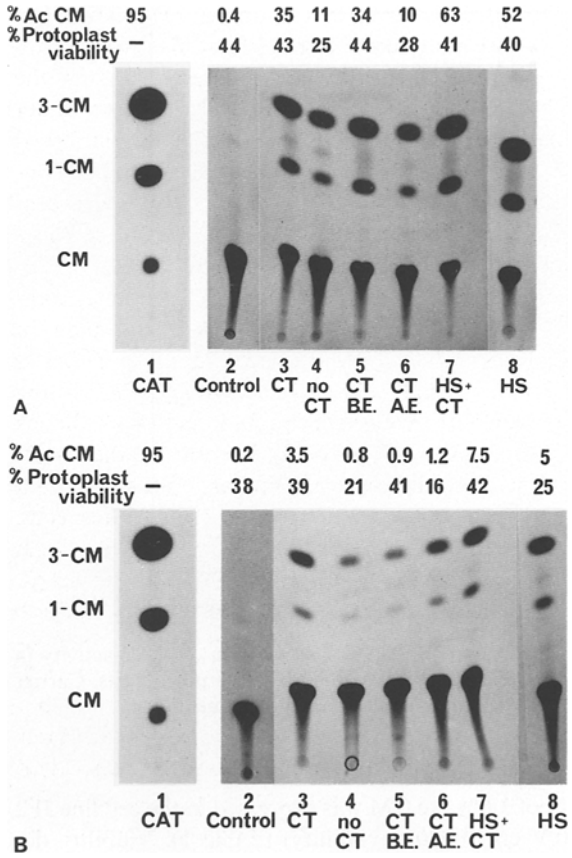

Fig. 2. **A** Effect of temperature and heat shock on black spruce protoplasts (BSF). BSF protoplasts were electroporated at 250 V cm^{-1} (960 μF) with 100 μg ml^{-1} pCaMVCN and 50 μg ml^{-1} carrier DNA. The autoradiogram of a TLC plate shows: CM = [^{14}C]-chloramphenicol, 1-CM = 1-[^{14}C] acetyl chloramphenicol, 3-CM = 3-[^{14}C] acetyl chloramphenicol; *lane 1:* CAT assay with commercial enzyme; *lane 2:* electroporated BSF protoplasts without plasmid; *lane 3:* CT = cold treatment (10 min ice before and after electroporation); *lane 4:* no CT = no cold treatment; *lane 5:* CT B.E. = cold treatment before electroporation (10 min on ice before electroporation); *lane 6:* CT A.E. = cold treatment after electroporation (10 min on ice after electroporation); *lane 7:* HS + CT = heat shock + cold treatment (10 min incubation on ice before and after electroporation); *lane 8:* HS = heat shock with no cold treatment. Percent protoplast viability is the relative viability within treatments. **B** Effect of temperature and heat shock on jack pine protoplasts (JP2). JP2 protoplasts were electroporated at 300 V cm^{-1} (960 μF) with 100 μg ml^{-1} pCaMVCN and 50 μg ml^{-1} carrier DNA. See **A** for description of lanes of TLC autoradiogram. Percent protoplast viability is the relative viability within treatments

levels of sonicated calf thymus carrier DNA during electroporation of this species. The addition of 0, 50, or 150 μg ml^{-1} carrier DNA to JP2 protoplasts (in the presence of 0, 50, or 100 μg ml^{-1} plasmid DNA) did not result in any increased CAT expression (Table 2).

Table 2. Effect of carrier DNA on CAT activity in jack pine protoplasts

Carrier (μg ml^{-1})	Plasmid[a] (μg ml^{-1})	% Ac CM[b]
0	0	0.2
50	0	0.2
0	100	2.2
50	100	2.8
50	50	1.8
150	50	1.8

[a] pCaMVCN
[b] % Ac CM is the cpm CM acetylated/cpm total CM (per 10^6 protoplasts). Voltage was 300 V cm^{-1} (960 μF)

Table 3. Effect of promoter and conformation of DNA on CAT activity

Plasmid[a]	Promoter	Conformation	% Ac CM[b]	
			Black spruce	Jack pine
pCaMVCN	35S	circular	35.0	4.2
pNCN	NOS	circular	37.0	4.0
pNCN	NOS	linear	28.0	10.0

[a] Plasmid was added at 100 μg ml^{-1} and carrier DNA at 50 μg ml^{-1} in all treatments
[b] % Ac CM is the cpm CM acetylated/cpm total CM (per 10^6 protoplasts). Voltage was 300 V cm^{-1} (960 μF)

Effect of promoter and plasmid conformation

The relative effectiveness of CAT driven by the pCaMVCN 35S promoter or the pNCN NOS promoter was determined in black spruce and jack pine protoplasts. Results showed that there was no observable difference between these promoters for CAT gene expression (Table 3).

Using 100 μg ml^{-1} of plasmid, linearized pNCN gave 2.5 times (10% Ac CM) higher levels of CAT activity than circular pNCN (4% Ac CM) in jack pine protoplasts (Table 3). In black spruce, linear pNCN gave slightly decreased (28% Ac CM) CAT activity as compared to circular (37% Ac CM).

Effect of temperature

Results with black spruce and jack pine protoplasts showed that cold treatment before and/or after electroporation had a detectable influence on CAT expression and protoplast viability (Fig. 2). In black spruce, high CAT expression was observed if protoplasts were incubated for either 10 min on ice both before and after electroporation (35% Ac CM; 43% viability), or 10 min

on ice only prior to electroporation (34% Ac CM; 44% viability). Conversely, if protoplasts were held on ice for 10 min only following electroporation, or incubated completely without ice, viability of protoplasts decreased and lower CAT activity was measured (approximately 10% Ac CM) (Fig. 2A). Results for jack pine showed that there was a decrease in CAT activity in all cases, unless protoplasts were incubated on ice both before and after electroporation (Fig. 2B).

Applying a 45 °C heat shock to protoplasts for 5 min prior to electroporation enhanced CAT activity for both species (Fig. 2). In black spruce, heat shock resulted in 63% Ac CM with cold treatment, and 52% Ac CM without cold treatment (Fig. 2A). In both cases, there was a slight decrease in protoplast viability. In jack pine, heat shock resulted in 7.5% Ac CM with and 5% Ac CM without cold treatment. Although protoplast viability decreased in jack pine if heat shock was not followed by cold treatment, this only slightly affected CAT activity (Fig. 2B).

Discussion

Results showed that a 250 and 300 V cm^{-1} (160–170 ms; 960 μF) electric pulse applied to black spruce and jack pine protoplasts, respectively, gave acceptable protoplast survival and the maximum transient CAT activity. These conditions are similar to those used by Bekkaoui et al. (1988) for transient gene expression in white spruce protoplasts. For comparison 500 V cm^{-1} was used for transient expression in electroporated alder protoplasts (at 960 μF) (Seguin and Lalonde 1988), and in Douglas fir and loblolly pine protoplasts (at 500 μF) (Gupta et al. 1988). According to Bates et al. (1988), the most important experimental parameters for electroporation are the pulse duration and the voltage. With black spruce and jack pine, optimization of voltage for each species and cell line was necessary to achieve the highest degree of electroporation efficiency (i.e., level of transient gene expression relative to viability). This was most evident in cell lines of jack pine, which differed in their voltage requirements. Variations in CAT activity were also demonstrated by the black spruce cell lines, three of which showed poor levels of transient gene expression under all the conditions tested. The importance of selecting optimal cell lines was also recently observed by F. Bekkaoui et al. (unpublished results) for gene expression in white spruce protoplasts.

The effects of plasmid concentration on CAT activity in black spruce and the effect of carrier DNA on CAT activity in jack pine are in agreement with other authors. Wilson et al. (1989) obtained 70% conversion to Ac CM at 150 μg ml^{-1} pCaMVCN using PEG-mediated uptake in white spruce protoplasts, and Seguin and Lalonde (1988) found no effect of carrier on transient expression of GUS (β-glucuronidase) in electroporated alder protoplasts. Results on CAT expression when driven by the pCaMVCN 35S promoter or the pNCN NOS promoter are similar to those of F. Bekkaoui et al. (unpublished results), who compared the effects of different CAT constructs on transient expression in white spruce protoplasts, in addition to protoplasts from the same cell lines of black spruce and jack pine presented here.

The use of linearized pNCN resulted in higher transient CAT activity than circular pNCN in jack pine. In several species, linear DNA has been found to be better than circular for plasmid uptake and integration leading to increased gene expression (Shillito et al. 1985; Ballas et al. 1988; Bekkaoui et al. 1988). In contrast, the results obtained with black spruce are similar to those reported in carrot protoplasts (Boston et al. 1987; Bates et al. 1988), tobacco protoplasts (Okada et al. 1986), and PEG-treated white spruce protoplasts (Wilson et al. 1989). The results observed in black spruce may indicate that the conformation of the pNCN plasmid is not critical for its transient expression under the conditions used.

Results for black spruce and jack pine showed that the uptake of plasmid pCaMVCN at low temperatures (i.e., ice before and after electroporation) was better than that obtained at room temperatures (i.e., no ice). The effect of cold treatment on transient gene expression using electroporation may be due to its influence on the physical properties of the protoplast membrane and the duration of the permeabilized state (Morikawa et al. 1988; Shigekawa and Dower 1988). This effect has been shown to vary, depending on the species being used (Potter et al. 1984; Langridge et al. 1985; Stopper et al. 1987; Bates et al. 1988).

The application of a specific heat shock was found to increase transient gene expression in black spruce and jack pine. Heat shock treatment was also shown to be beneficial in non-coniferous plant material, for example, tobacco protoplasts (Shillito et al. 1985), alder protoplasts (Seguin and Lalonde 1988), and rice protoplasts (Zhang and Wu 1988). In contrast, in carrot protoplasts, a heat shock did not substantially improve CAT transient gene expression (Boston et al. 1987; Bates et al. 1988). Thompson et al. (1987) suggested that heat shock may have an effect on the permeabilized state of the protoplast membrane. In addition, the production of protective heat shock proteins may also play a role (Key et al. 1981).

In conclusion, the results presented support the view that electroporation is usable with a number of different coniferous species. Further, they show that there is a range of intra-species variation in transient gene expression which predicates the need to experiment with several genotypes. Further investigation is needed to determine the factors which cause enhanced CAT expression in

black spruce and jack pine protoplasts after heat shock treatment. Experiments are now aimed at obtaining stable transformation and plantlet regeneration from transformed protoplasts.

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