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Transient expression of the β-glucuronidase gene in embryogenic callus of *Picea mariana* following microprojection

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Abstract. A microprojection protocol using the DuPont Biolistic™ particle delivery system and the β-glucuronidase (GUS) reporter gene fused with the 35S promoter of Cauliflower mosaic virus (CaMV) was developed for Picea mariana callus. Comparison of four tungsten microprojectile sizes showed the highest transient gene expression with 1.11μm diameter particles. Adsorption of DNA on the microcarriers using calcium chloride led to higher GUS gene activity than using polyethylene glycol. GUS gene activity in P. mariana was the highest when cells were treated 5 and 6 days after subculturing to fresh media. The wheat ABA-inducible Em gene promoter yielded 4.5 times higher GUS gene activity than the 35S CaMV promoter. Comparison of transient GUS gene expression among 10 P. mariana embryogenic cell lines from six different open-pollinated families showed comparable gene activity, with the exception of one family showing no GUS gene activity.

Introduction

Recent advances in genetic transformation of plants have made possible the transfer of chimeric genes into the genome of forest trees. It is expected that this technology will help circumvent some of the difficulties of forest tree improvement associated with classical breeding programs (Cheliak and Rogers 1990; Wilson et al. 1989).

Several transformation techniques have been used with varying degrees of success in gymnosperms (Sederoff and Stomp 1990). However, transgenic conifers have yet to be recovered. While it is now clear that the delivery of DNA into conifer cells is feasible, the obstacles to achieving stable integration have to be identified.

Although infection by Agrobacterium was reported for nearly 60 conifer species (Sederoff and Stomp 1990), attempts to use this organism for conifer genetic transformation have been unsuccessful. Transient gene expression of foreign genes in conifer protoplasts using electroporation was reported for seven species (Charest et al. 1991; Sederoff and Stomp 1990) whereas use of microprojectile-mediated DNA transfer was reported for Picea glauca (Ellis et al. 1991), Pinus taeda (Stomp et al. 1990), and Picea abies (Yibrah and Clapham 1990).

Recent results from several laboratories demonstrated the feasibility of clonal propagation in conifers through somatic embryogenesis, in species of Pinus (Gupta and Durzan 1986), Picea (Attree et al. 1989; Cheliak and Klimaszewska 1990; Hakman and von Arnold 1985), Larix (Klimaszewska 1989; von Aderkas et al. 1990), Pseudotsuga (Durzan and Gupta 1987), and Abies (Schuller et al. 1989). One potential scenario for obtaining transgenic conifers is the establishment of transgenic embryogenic cell lines, from which plants would be regenerated via somatic embryogenesis.

The objective of this research was to investigate the physical and biological factors controlling transient GUS gene activity in embryogenic cell lines of P. mariana.

Materials and methods

Plant materials. 10 embryogenic cell lines of P. mariana (Mill.) B.S.P. from seeds of six open-pollinated P. mariana families were used in this experiment and were maintained on modified LM medium (Cheliak and Klimaszewska 1991) at 25°C in the dark with subculturing every 14 days. These cultures were established previously from excised mature embryos (Cheliak and Klimaszewska 1991). Cell suspensions of Nicotiana tabacum L. var. Petit Havana line SR1 (Maliga et al. 1973) were maintained in Murashige and Skoog liquid medium (Murashige and Skoog 1962) with 0.5 mg/L 2,4-D and transferred to fresh medium every 7 days.

DNA preparation for microprojection. Plasmids pRT99GUS and pBM113Kp were isolated according to the method of Tartof and Hobbs (1987). Plasmid pRT99GUS (6.7 kb) contains genes encoding for β-glucuronidase (GUS) and neomycin phosphotransferase II which are flanked by the promoter and

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terminator sequences of the 35S gene of CaMV (Töpfer et al. 1988). Plasmid pBM113Kp (5.4 kb) contains the GUS gene flanked by the ABA-inducible promoter from the early methionine (Em) protein gene of wheat and the CaMV terminator sequences (Marcotte et al. 1988). The GUS gene was used as a reporter gene because of its efficiency as well as the simplicity involved in the quantification of results.

Microprojection experiments. DNA transfer was carried out using the Biolistic™ particle delivery system PDS-1000 (DuPont, Wilmington DE) following the manufacturer's recommendations. For microprojection, cells (150-500 mg fresh weight) were weighed under sterile conditions and placed in the centre of 9 cm diameter Petri dishes containing fresh medium (approximately 20 mL). For each bombardment, 1 µg DNA was delivered. Samples were placed 12.5 cm from the stopping plate under a vacuum of 800 mm Hg. All experiments were replicated five times and repeated at least three times.

To optimize the DNA precipitation procedure, cell line R4F14 was bombarded with plasmid pRT99GUS adsorbed to tungsten particles using the calcium chloride precipitation method developed by Klein et al. (1987) or by a polyethylene glycol (PEG) method (communicated by DuPont). For PEG precipitation, 10 µg DNA (1 µg/µL) was added to 2.5mg tungsten particles (DuPont) in 150 µL distilled water. 20µL 40% PEG-4000 (Sigma, St-Louis, MO) was added followed by 5 µL 0.1M spermidine (Sigma, free base) and the solution incubated at room temperature for 10 min. The supernatant (150µL) was discarded and the remainder was used for microprojection. To determine optimal particle size for DNA delivery, calli of cell line R4F14 were bombarded with DNA adsorbed on particles with average sizes of 0.36, 0.73, 1.11, or 1.3µm (DuPont).

GUS assays. After bombardment the Petri dishes containing calli were incubated at 25°C in the dark for 2 days. The cells were then transferred to 600µL modified GUS reaction buffer without mannitol (Jefferson et al. 1987) modified with the addition of 0.1 % Triton X-100 (Sigma) and incubated at 37°C in the dark for 2 days before examination using a Nikon-diaphot inverted microscope. Quantitative determination of GUS activity was carried out by determining the number of "expression units" per sample. This term is defined as the number of islands of cells that showed a blue colouration regardless of the number of blue cells in these islands. A similar approach was used in other studies (Sanford 1990, Ellis et al. 1991) although it is recognized that this assay may underestimate results (Sanford 1990). To ascertain proper comparison of results, data were analyzed either in terms of expression units per 100mg tissue dry weight (d.w.), expression units per 100mg tissue fresh weight (f.w.), or expression units per 100µg tissue proteins. However, all three methods of expressing results led to comparable conclusions, and so the results reported here are expressed only in terms of expression units per 100mg tissue f.w.

Results

To obtain proper gene delivery using the Biolistic™ particle accelerator the effect of four tungsten particle sizes and two DNA precipitation methods were evaluated with P. mariana embryogenic cell line R4F14 in combination with plasmid pRT99GUS.

GUS gene activity in cells, microprojected with plasmid DNA adsorbed to tungsten particles using the $CaCl_2$ precipitation method, was greater than GUS gene activity when PEG was used to precipitate DNA (Fig. 1a). Particle size also affected transient GUS gene activity in R4F14 cells (Fig. 1b), with 1.11µm diameter particles yielding the highest GUS gene activity. Microprojectile bombardment led to GUS gene activity in single cells as well as in clusters of cells as shown in Fig. 2. There was no correlation between cell size and GUS gene activity (data not shown). Tobacco cells bombarded with plasmid pRT99GUS yielded a level of GUS gene activity 2.5 times greater than the highest level observed in P. mariana (Fig. 3).

Biological factors were evaluated to further optimize this method of delivery. The time of bombardment after subculture affected GUS gene activity in cell line R4F14 (Fig. 4a). GUS gene activity was greatest in 5- and 6-day-old cells, as it was 4-12 times higher than at other times during the culture cycle. These two days coincide with the time during the growth cycle when approximately half of the cell biomass accumulates (Fig. 4b).

The effect of the genetic background of the recipient cells was investigated by comparing transient GUS gene activity in 10 embryogenic lines originating from six different open-pollinated families.The data indicated little variation among the lines since all, with the exception of one, were within one standard deviation (Fig. 5). However, one cell line (R17F20) did not yield any transient gene expression.

The third biological parameter investigated was the effect of promoter sequence on GUS gene activity. Preliminary studies showed that a monocot promoter from the ABA- inducible EM gene of wheat (Marcotte et al. 1988) was strongly expressed in conifers (G. Pilate and D.I. Dunstan, personal communication). In the present investigation GUS gene activity was 4.5 times greater in cells bombarded with plasmid pBM113Kp (ABA-inducible) than in cells bombarded with plasmid pRT99GUS (35S promoter) (Fig. 3).

Discussion

Techniques for genetic transformation of conifers are still under development because no transformation system has yet proved to be reproducible. Unlike Agrobacterium-mediated DNA transfer, microprojection is not limited by host range restrictions. Also it is relatively fast and can be applied to various kinds of plant material such as tissue cultures or mature tissues. In P. mariana embryogenic callus cultures microprojection and gene expression were shown to be both rapid and fairly reproducible despite high standard variation of the results. The technique is of particular importance to research on gene function in conifers where the study of tissue or cell specific gene expression is greatly limited by their long generation times. This is of particular significance to studies on gene induction during flowering.

The method of precipitation and the size of the tungsten microprojectiles were two important factors of microprojection. For the line R4F14, the $CaCl_2$ method and a tungsten particle size of 1.11µm gave the highest transient gene expression with the plasmid

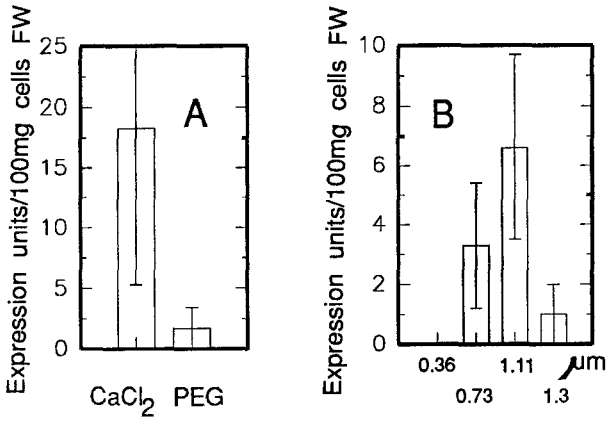

Fig. 1A. GUS gene activity in 5-day-old R4F14 cells after microprojection with tungsten particles treated with calcium chloride ($CaCl_2$) or polyethylene glycol (PEG). Fig. 1B. Effect of tungsten particle size on GUS gene activity in 5-day-old R4F14 cells of P. mariana. Error bars indicate standard deviation.

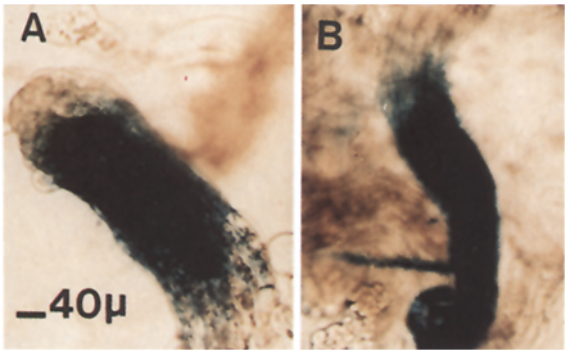

Fig. 2. GUS gene activity in 5-day-old P. mariana R4F14 cells after microprojectile bombardment showing (Fig. 2A) GUS gene expression in the meristematic region of a proembryo and (Fig. 2B) in the suspensor region of another proembryo.

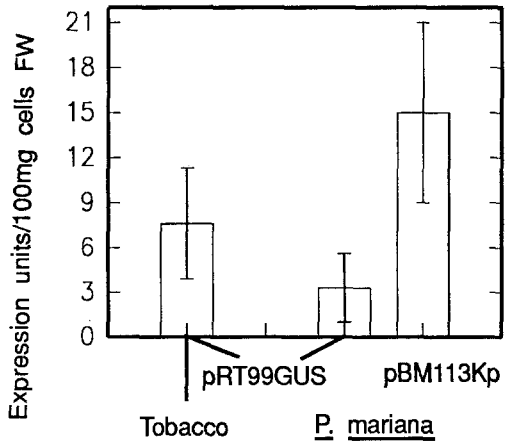

Fig. 3. Comparison of 35S CaMV (pRT99GUS) and wheat ABA-inducible (pBM113Kp) promoters on GUS gene activity in 5-day-old R4F14 cells of P. mariana. GUS gene activity in N. tabacum cell supension bombarded with pRT99GUS is given as a control. Error bars indicate standard deviation.

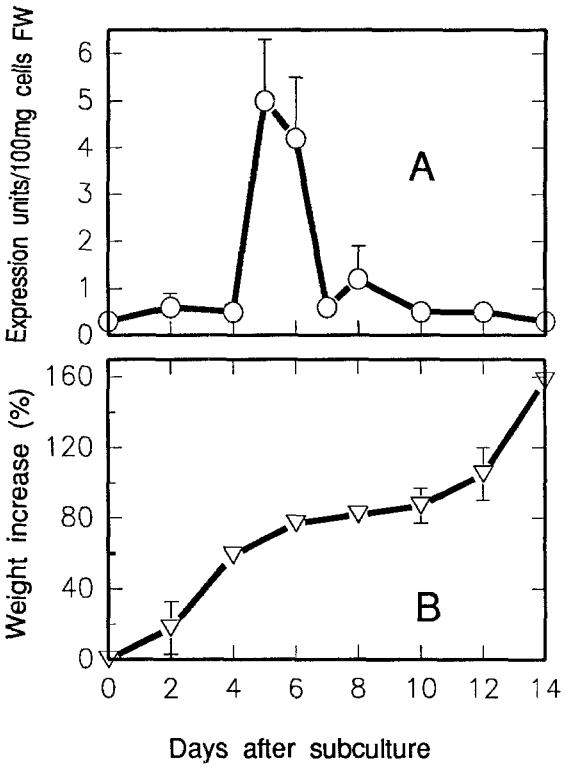

Fig. 4. Effect of cell age on GUS gene activity (Fig. 4A), and cell growth curve (Fig. 4B) of R4F14 cells. Error bars indicate standard deviation.

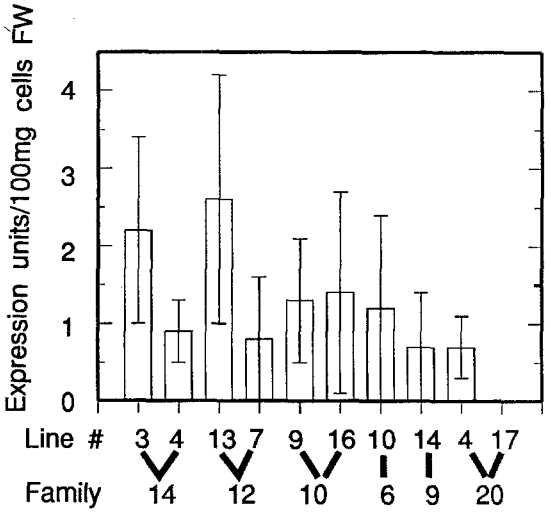

Fig. 5. GUS gene activity in 10 embryogenic cell lines (12 day old cells) of P. mariana. Error bars indicate standard deviation.

pRT99GUS. It has been reported that optimal tungsten particle size should be a tenth of that of recipient cells (communicated by DuPont). However, measurements carried out on P. mariana cell line R4F14 show an average cell size of approximately 70µm x 10µm for suspensor cells and approximately 20µm x 10 µm for meristematic cells (unpublished results).

It was observed that 5- and 6-day old cells display greater transient expression than younger or older cells. A similar phenomenon was reported in tobacco and sunflower protoplasts after electroporation (Nagata et al. 1987; Kawata et al. 1990). These authors concluded that the 35S promoter is cell cycle specific because it is mostly expressed during the S phase of the cellular cycle of (Nagata et al. 1987, Kawata et al. 1990). Further work is needed to assess the cell cycle influence on transient expression of the 35S promoter in P. mariana calli since cells are non-synchronous in this system. In contrast to Ellis et al. (1991) who observed transient GUS activity to be limited to proembryonal heads in embryogenic calli of Picea glauca, our results showed GUS gene expression in non-organized cell clusters as well as in suspensor cells.

The genetic background of the recipient cells had little effect on GUS gene activity since only one out of the 10 lines investigated did not yield any transient expression. Tautorus et al. (1989) observed significant differences in transient gene activity between three cell lines of P. mariana after electroporation. It would be interesting to determine why cell line R17F20 did not show GUS gene activity. There was no correlation between GUS gene activity and either the rate of cell growth or embryo production (data not shown). Moreover, cell line R4F14 produced approximately twice as many somatic embryos as other cell lines (data not shown).

Promoter sequences were found to influence transient gene expression. The ABA-inducible promoter of the wheat Em protein, a monocot promoter, was more effective than the 35S promoter which is constitutive in dicots. Moreover, preliminary results show this promoter to be ABA-inducible in conifers. In contrast, transient gene expression driven by the Em promoter was weak in cell suspension cultures and leaf discs of the angiosperm Populus deltoides x nigra whereas the 35S promoter was expressed at high levels in both these tissues (Y. Devantier, personnal communication). Two monocot promoters from the maize ADH and PEP carboxylase genes fused to the GUS reporter gene yielded lower levels of transient gene expression than the 35S promoter with Picea glauca (Ellis et al. 1991). Transient gene expression studies using electroporation in spruce have highlighted the importance of various promoter sequences (Bekkaoui et al. 1990, Yibrah and Clapham 1990).

This work describes the first transient gene expression in embryogenic callus cultures of P. mariana using microprojection as the delivery method. Others reported transient GUS and chloramphenicol acetyl transferase activity in protoplasts of P. mariana using electroporation for gene delivery (Bekkaoui et al. 1990; Tautorus et al. 1989). We speculate that stable transformation of P. mariana embryogenic cells may be achieved through the use of strongly activated promoters. In tobacco and corn, a relative proportion of 2-5% of cells showing transient gene expression yields stable transformed tissues after microprojection (Sandford 1990). Thus, an increase in gene promoter activity in P. mariana could facilitate the recovery of transgenic tissues by providing higher resistance to selectable markers under use. For this, other plant promoters are being evaluated.

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