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Protocols

Efficient Preparation of Maritime Pine (*Pinus pinaster)* Protoplasts Suitable for Transgene Expression Analysis

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Abstract. Establishment of protoplast techniques for conifers is of crucial importance to forest biotechnology. Protoplasts can be used to regenerate whole trees and to study transcriptional regulation in woody plants by promoter analysis. Here we describe a technique for isolating protoplasts from *Pinus pinaster* shoots. Protoplast viability was determined by fluorescein diacetate staining. Transient gene expression was studied after electroporation of protoplasts with plasmid DNA containing a β-glucuronidase (*GUS*) reporter gene. The 35S promoter and a pine (cytosolic glutamine synthetase) promoter both were able to drive *GUS* expression, indicating applicability of conifer protoplasts as a model to study gene promoters using transient expression.

Introduction

Protoplasts provide a powerful tool for evaluating marker gene expression in plants. Gene expression systems that allow direct comparison between different promoters in plants under transient conditions can be used for a wide range of applications in plant molecular biology. In conifers, protoplasts also have been isolated from seedling materials (embryos, cotyledons) preconditioned *in vitro* by culture on a medium-containing auxin and cytokinin prior to protoplast isolation (David et al., 1982).

Although protoplasts have been isolated from haploid cells of conifers, including pollen of *Cupressus arizonica* (Duhoux, 1980) and female gametophytes of *Picea abies* (Hakman et al., 1986), most of the efficient preparation methods use suspension cultures as starting material. Protoplasts have been isolated from pine seedling cotyledons (Patel et al., 1984) and roots (Faye and David, 1983), but

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seedling shoots have not been used as starting material. We now present a method to prepare protoplasts from shoots of maritime pine. The protocol also can be used to prepare protoplasts from cotyledons, but initially it was developed to evaluate the promoter activity of a pine *GS1* gene expressed in the vascular tissue. The method can be applied more generally for evaluation of transcriptional regulation of conifer genes.

Materials and Methods

Plant material

Maritime pine seeds (*P. pinaster*) used in all experiments were from INFOCA (Spain). Seed germination and growth of the seedlings were as described previously (Cánovas et al., 1991).

Protoplast isolation

Shoots from 12- to 15-day-old seedlings were used as the starting material. Enzyme penetration was facilitated by splitting the shoot fragments longitudinally 2-4 times and then cutting transversely into 2- to 5-mm thick pieces. Mesophyll and vascular cells were released by incubation overnight in the dark at 25ºC in an enzyme mixture (0.4% [w/v] cellulase [Calbiochem], 0.4% [w/v] macerase [Calbiochem], 0.4% sucrose, 0.44% [w/v] K3 medium) and 1 g of tissue in 10 mL of enzyme solution.

Undigested materials and cell debris were removed by filtration through a 75-μm mesh sterile metallic sieve and washed after gravity sedimentation with a solution containing 0.4 M of sucrose and 80 mM of KCl.

Sedimentation and washes were repeated 2 times, and the protoplasts were resuspended to a 1.10^5/mL final density in the electroporation medium (Shen et al., 1992). Protoplast viability was determined by staining with fluorescein diacetate (Rotman and Papermaster, 1966).

Electroporation

The protoplast suspension (1 mL) was mixed with 50 μg of supercoiled plasmid DNA and 10 μg of sheared salmon sperm DNA as a carrier and electroporated in a pulser^{tm} cuvette (BioRad) with a 0.2-cm electrode. A capacitor discharge system was used to deliver 800 V/cm. After the electrical discharge, the protoplasts were left at room temperature for 10 min and then diluted with a volume of 0.5 M mannitol, 88 mM sucrose, and 0.5 mg/L carbenicillin. The protoplasts were cultured in the dark at 24ºC for 36 h.

β-*glucuronidase assay*

Cultured protoplasts were recovered by sedimentation. Pellets were resuspended in the grinding buffer (Jefferson et al., 1987) containing 3.3% polyvinylpyrrolidone. Homogenization was performed in Eppendorf tubes containing sand and using plastic micropestles.

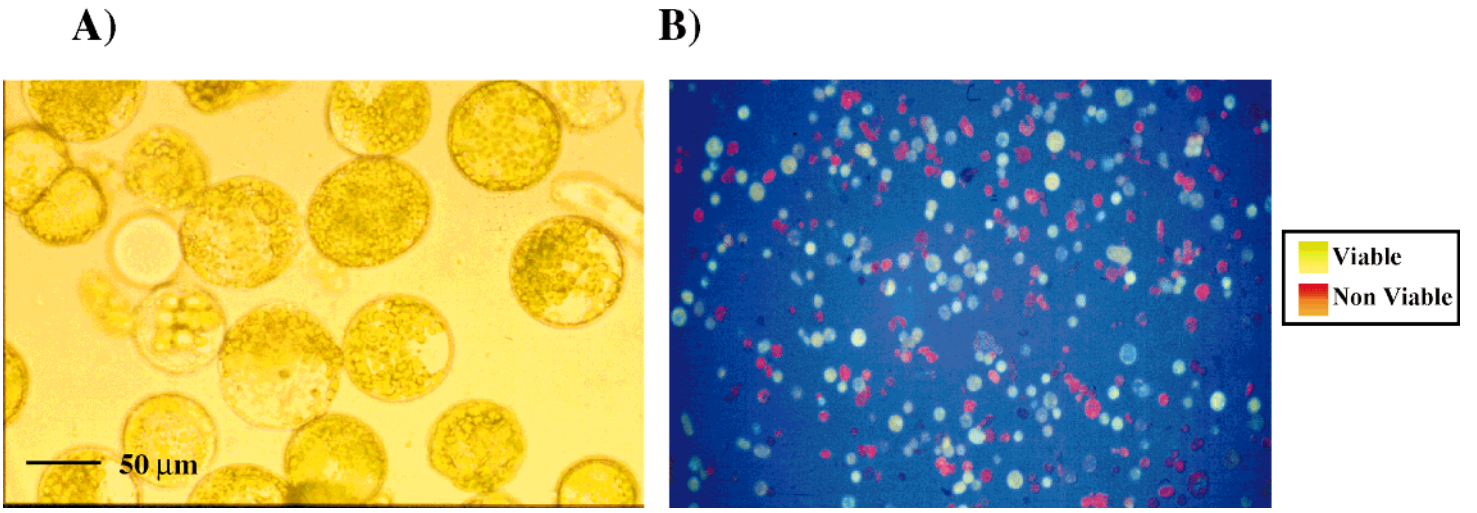

Figure 1. (A) Protoplasts isolated from shoots of 10-day-old pine seedlings (*Pinus pinaster*). (B) Fluorescence micrograph of pine protoplasts stained with fluorescein diacetate. Scale bar represents 50 µm.

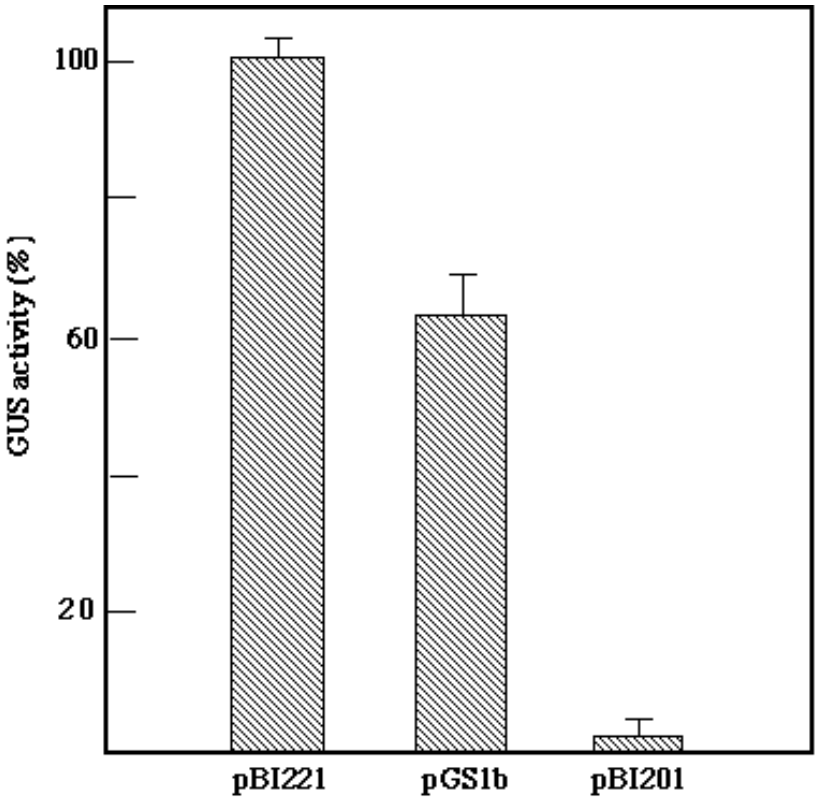

Figure 2. Fluorimetric determination of GUS activity in extracts of *P. pinaster* protoplasts 24-36 h after electroporation with 50 μg of DNA. The average values of 3 independent experiments were used to calculate GUS activity for each construct. The values were normalised using the negative control. Bars indicate standard deviations. Negative control (-) = PBI 201 promoterless plasmid. Positive control = pBI 221 plasmid with 35S promoter.

The samples were cleared by centrifugation at 13,000 *g* for 30 min and the supernatants were passed through a spun column containing Sephadex G-25.

Protein concentration was determined by the Bradford procedure (1976). The β-glucuronidase activity was determined using a fluorometric assay (Jefferson et al., 1987). Before the assay, the protein concentration of the supernatant was adjusted to 1 mg/mL, 75 μL of the extract was added to 500 μL of grinding buffer containing 1 mM 4-methylumbelliferyl glucuronide, and the solution was prewarmed to 37ºC. The reactions were carried out at 37ºC, and the fluorescence was measured at 15-min intervals using a FL 6000 fluorescence Reader Bio-TER (excitation 365 nm, emission 455 nm) as described (Jefferson et al., 1987).

Results and Discussion

Protoplast isolation

During our studies, we arrived at several conclusions regarding protoplast isolation from conifers. The use of desalted pectinase, hemicellulase, and cellulase did not increase yield or viability in the preparation. However, the ratio between time and enzyme concentration greatly affected the number and quality of protoplasts. Lower enzyme concentration and longer incubation times (usually overnight) gave us the best results, as described in Materials and Methods.

We tried several techniques for removing undigested materials and cell debris. Ficoll density gradient centrifugation has been used to obtain clean conifer protoplast pellets (Gupta and Durzan, 1986). However, the technique was useless in our hands. In addition, the fragile *P. pinaster* protoplasts, which have high densities because of increased starch content, were disrupted by the usual centrifugation procedures. It is advisable, so far, to allow protoplasts to settle in tubes without centrifugation.

With the above in mind and using the protocol described in Materials and Methods, we prepared protoplasts of 80-90% viability as determined by staining with fluorescein diacetate (Rotman and Papermaster, 1966) (Figure 1b) and direct cell count in a hemocytometer.

Gene expression in electroporated protoplasts

We studied transient expression of 2 promoters using pine seedling shoots as the main target tissue. The following promoter-*GUS* vector constructs were used:

- pBI221 - 35S promoter from cauliflower mosaic virus (Mitsuhara et al., 1996)
- GS1b - Pine GS1b promoter (Gómez-Maldonado et al., unpublished results)
- PBI201 - Promoterless negative control, plasmid carrying only *GUS*

Each plasmid was introduced by electroporation into pine protoplasts prepared as described above. The protoplasts were assayed 24-36 h later for GUS activity. Construct PBI221 (35S promoter) displayed 33% higher GUS activity than the pine promoter GS1b construct; the negative control pBI202 construct gave only basal activity (Figure 2). We are interested in the *GS1b* gene whose expression is associated with vascular tissue in pine seedlings (Avila et al., 2001). The shoot protoplasts provide a system to evaluate the upstream promoter regions of this gene and to identify any *cis*-acting elements.

We have established a rapid transient assay for functional analysis of promoters in conifers. We can use this procedure to analyze the *GS1b* promoter and other conifer promoters on a transient expression basis without the need for heterologous systems. Deletion analysis of the *GS1b* promoter is currently in progress.

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