PROGRESS WITH SOMATIC EMBRYOGENESIS , CRYOPRESERVATION, AND TRANSFORMATION OF SLASH PINE

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Abstract:-- Slash pine *(Pinus elliottii)* is an important sustainable fiber species in the Southern USA. Genetic improvement of slash pine could be augmented by the clonal forestry options that may arise from the successful application of somatic embryogenesis. In turn, somatic embryogenesis offers the only route currently available for genetic transformation of pines. We report progress towards developing and improving protocols at the University of Georgia.

INTRODUCTION

Protocols for regeneration of *Pinus elliottii* have been published (Liao and Amerson, 1995a, 1995b; Newton *et al.,* 1995; Smith, 1996) and transformation by particle bombardment leading to regeneration of transgenic plants has been claimed (Walter *et al.,* 1998b). A reliable somatic embryogenesis and cryopreservation protocol is essential for transformation and the evaluation of the effect of introduced genes. We report progress towards establishing a pine transformation and regeneration capacity at the University of Georgia.

METHODS

Initiation and maintenance of cultures

Immature cones from 4 slash pine clones were kindly provided by International Paper for this study. Embryogenic tissue was obtained from seed explants, comprising the megagametophyte and the enclosed immature zygotic embryos, placed in culture in June (696 explants) and July (900 explants). Explants were placed on DCR (Gupta and Durzan, 1985) or half-strength P6 medium (Teasdale *et al.,* 1968) and maintained on the same medium subsequently with subculture at 2-weekly intervals. In December 1998, all established embryogenic cell lines were evaluated using a dissecting microscope. The presence of bullet-stage somatic embryos was noted, and cell lines were allocated to the following classes: 1, likely to produce high-quality mature somatic embryos; 2, likely to produce abnormal mature somatic embryos; and 3, unlikely to produce mature somatic embryos. All class 1, and a sample of class 2 and 3 cell lines were transferred to EDM6 medium (Walter *et al,* 1998a). After a month or more on EDM6, tissue samples were transferred to EDM, EMM1, or EMM2 media (Smith, 1996). Mature somatic embryos were transferred to germination media (Smith, 1996).

Cryopreservation

Tissue of 9 embryogenic cell lines that had been maintained on EDM6 for at least 1 month was suspended (1 g / 5 ml) in EDM6 liquid medium containing 0.4 M sorbitol (Hargreaves and Smith, 1992) and incubated overnight on an orbital shaker. The following day suspensions were chilled to 4° C and an equal volume of chilled EDM6 with 0.8 M sorbitol and 20% DMSO added. Aliquots of 1 ml were transferred to Nunc cryovials which were then placed in a Mr. Frosty (Nalgene) which had been pre-chilled overnight to –20°C. The Mr. Frosty was then placed in a –80°C freezer for 1.5 hours, after which the cryovials were placed into liquid nitrogen in the vapor phase. A 1 ml sample of each cell line in the

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sorbitol / DMSO solution was plated onto EDM6 medium after rinsing according to the Hargreaves and Smith protocol. Twenty-four hours later, one sample of each cell line was recovered from liquid nitrogen, using the methods of Hargreaves and Smith. Growth of the cryostored and control cell lines was compared following determination of the tissue fresh weight after 6 weeks.

Transformation by particle bombardment

Tissue from several slash pine embryogenic cell lines was plated on filter paper according to the protocol of Walter and co-workers (Walter *et al,* 1994, 1998a) Transient expression of the *uidA* reporter gene was determined three days after bombardment with gold particles coated with DNA prepared from the plasmid pBI426 (Bommineni *et al,* 1993).

RESULTS

Initiation and maintenance

Immature seeds from all four clones gave rise to a total of 301 embryogenic cell lines from both collection dates. Initiation frequencies, defined as the number of cell lines which were maintained until December 1998, ranged from 1.1% to 6.7% (mean for all trees, 3.9%) in the first collection period. In the second week of collection, the mean initiation frequency was 10.2%, with a range of 6.3% to 11.3%. DCR medium was more effective in the first collection (Table 1), while P6 medium was more effective in the second collection, however these differences were not statistically significant. The P6 medium gave rise to far more cell lines judged to be capable of producing mature somatic embryos (class 1, 2) than did the DCR medium (Table 1). This difference between media was significant ($P<0.05$), but source tree and collection date did not have significant effects.

A sample of 30 cell lines was submitted to the development and maturation protocols in May 1999. Mature somatic embryos have been recovered from 15 lines (all class 1) and germination of some mature embryos has been recorded in July 1999. These studies are continuing.

Cryopreservation

All 9 cell lines recovered from liquid nitrogen showed renewed growth within 3 days of being placed on EDM6 medium. After 6 weeks, the mean tissue fresh weights were 3.03 g for the non-frozen control tissue, and 2.76 g for the tissue that was stored for 24 hours in liquid nitrogen. The mean tissue weights were not significantly different.

Table 1. Slash pine initiation and embryo quality.

	DCR medium		**P6 medium**	
Mean initiation frequency				
June collection	5.1%		2.7%	
July collection	9.2%		11.2%	
Percent cell lines producing embryos of given class:				
Class 1	1.3%	(2 lines)	42.4%	(66 lines)
Class 2	14.5%	(22 lines)	28.4%	(61 lines)
Class 3	84.2%	(128 lines)	41.9%	(90 lines)

Particle bombardment

All slash pine cell lines tested to date showed transient expression of the *uidA* gene 3 days after bombardment. Tissue bombarded with pBI426 in February 1999 gave results as high as 1500 foci per gram fresh weight, which is consistent with the results published for radiata pine (Walter *et al.,* 1994). By July 1999, only one slash pine cell line continued to give such a high response, and most cell lines were giving values in the low hundreds. This is attributed to the tendency of slash pine embryogenic tissue to progress from primitive to advanced somatic embryos with serial culture on EDM6 medium. The tissue with transient expression had a range of responses from single cells to clusters of cells showing the dark blue stain. This response parallels the experience with transformation of slash pine in New Zealand (Walter and Smith, unpublished data: Walter *et al.,* 1994).

CONCLUSIONS

We have confirmed that the New Zealand protocols are effective for cryopreservation of slash pine, and for transient expression of genes introduced through particle bombardment. Ongoing work will focus on improvements to plant regeneration protocols and understanding the factors contributing to successful foreign gene integration.

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