DIRECT SOMATIC EMBRYOGENESIS FOR RAPID, COST EFFECTIVE PRODUCTION OF TRANSGENIC SUGARCANE (*SACCHARUM* SPP. HYBRIDS)

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Abstract

During the last decade, considerable effort has been expended in developing an efficient transformation system for sugarcane. Most protocols employ microprojectile bombardment of gene constructs into embryogenic sugarcane callus. The numbers of transgenic plants produced per bombardment range from 0.1-20 globally, with genotype response and selection regime appearing to be the most important factors influencing efficiency. The time taken to regenerate transgenic plants from callus via this route of indirect somatic embryogenesis is typically 24-36 weeks. We report a novel approach using a direct somatic embryogenic route of regeneration. The technique involves gene delivery to transverse explants derived from immature leaf roll pre-cultured for only 2 weeks on medium containing low levels (0.3 mg/l) of the auxin 2,4-D. Plantlets are ready for hardening off after 13-22 weeks. In a comparative study, PCR analysis showed that transformation efficiencies for the direct and indirect morphogenic routes were similar. Due to faster regeneration times and fewer subcultures, the cost of production via direct somatic embryogenesis can be reduced from R215 to R52 per transgenic plant.

Rationale

The use of embryogenic callus as target material for microprojectile bombardment has been used by sugar industries world wide in genetic engineering programmes for the production of transgenic sugarcane. Embryos have been induced to develop indirectly via an undifferentiated cell mass or callus stage from leaf discs or floral parts (Bower and Birch, 1992; Gallo-Meagher and Irvine, 1993; Snyman *et al*., 1996; Ingelbrecht *et al*., 1999). Despite this common approach, transgenic production ranges widely from 0.1 and 2.9 to 20 plants per bombardment (Snyman, unpublished results; Gallo-Meagher and Irvine, 1996; Bower *et al*., 1996, respectively). The choice of cultivar, genotypic responses to hormone treatment and selection regime may account for the differences in efficiency reported.

Aside from widely varying levels in transgenic production efficiency, the use of embryogenic callus as target material for bombardment has other limitations. Establishing, developing and maintaining callus cultures is labour intensive and the recovery of transgenic plants ready for glasshouse planting may take as long as 36 weeks (Bower *et al*., 1996). To minimise the time spent generating embryogenic callus, one approach would be to employ a route of morphogenesis from leaf discs which is faster than the indirect morphogenic route. In a study of cell suspension-derived protoplast regeneration, Aftab and Iqbal (1999) reported the formation of somatic embryos directly from the cut edges of young sugarcane leaf discs with very little intervening callus, so this route was seen to have the potential to speed up a transformation programme.

The aim of this study was to compare the efficiency of production of transgenic sugarcane regenerated via direct somatic embryogenesis from leaf roll discs with the conventionally used indirect morphogenesis via callus. The model transgene system chosen for the experimental work involved microprojectile bombardment of the *pat* and *nptII* genes, encoding herbicide (Buster) and antibiotic resistance respectively, the latter for the purpose of transgenic plant selection in culture.

Direct somatic embryogenesis in sugarcane

The formation of embryos directly on leaf discs was observed in our laboratory on medium (Murashige and Skoog, 1962) containing 0.3 mg/l 2,4-D. Leaf discs cultured for 2 weeks exhibited organised outgrowths on the cut surface of the discs, which by 3-4 weeks were clearly embryonic, with a well defined cellular structure (results not shown).

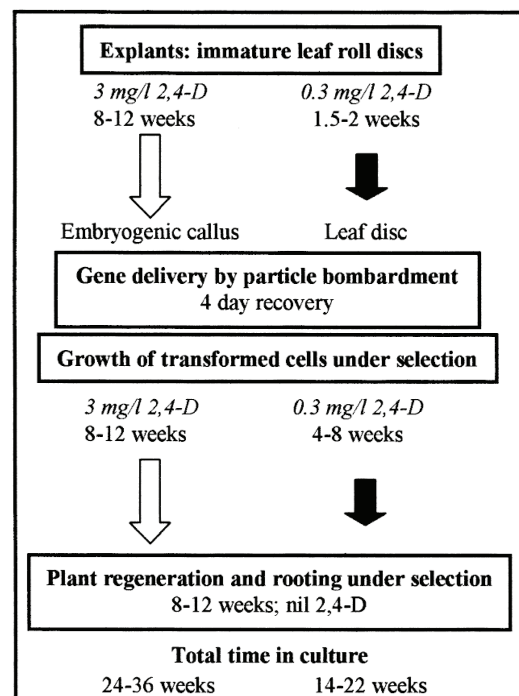

Figure 1. Relative time taken to produce transgenic sugarcane via direct and indirect morphogenic routes.

Table 1. A comparison of factors affecting the cost of transgenic sugarcane production via direct and indirect morphogenesis.

	Transformation approach	
	Indirect morphogenesis	*Direct morphogenesis*
Target material	Embryogenic callus	Immature leaf roll discs
2,4-D concentration (mg/l)	3	0.3
Number of subcultures	15	10
Time taken to generate plants (weeks)	24-36	13-22
Number of plants regenerated on selection medium (per bombardment)	0.1±0.04*	1.8±0.39*
Proportion of plants containing the *pat* gene (determined by PCR analysis)	100%	72%
Cost per transgenic plant (R)	**215**	**52**

* based on mean number of regenerated plants ± SE for varieties N12 and N19 (n=88-186 bombardments).

Relative time scale of regeneration: direct versus indirect embryogenesis

The feasibility of replacing embryogenic calli with embryogenic leaf discs from young sugarcane leaf rolls as target material for bombardment was assessed. The leaf roll discs were pre-cultured for 1-2 weeks and somatic embryo formation, albeit it at a slower rate on medium containing the selection antibiotic, occurred directly on discs 4-8 weeks after bombardment (Figure 1). Plantlet regeneration was observed 12-20 weeks after bombardment when the discs were transferred to regeneration medium (MS plus selection antibiotic, without 2,4-D), resulting in a total time saving of 10-14 weeks using the direct route. In addition, 1.8 plants per bombardment were regenerated, as opposed to 0.1 from the indirect morphogenic route (Table 1).

Comparative transformation efficiencies and production costs

A preliminary assessment of transformation efficiency of sugarcane plants regenerated via direct and indirect somatic embryogenesis was performed using specific PCR amplification of the *pat* transgene. PCR was carried out on leaf DNA extracts of a subset of regenerated plants. All the plants tested that regenerated via indirect embryogenesis were shown to contain the *pat* gene, whereas in plants regenerated via the direct route, only 72% of the plants were positive (Table 1). The higher percentage of nontransformed plants regenerated via the direct route may be due to rapid formation of somatic embryos and a concomitant short time spent under selection pressure. Future work will include increasing the concentration of the selection antibiotic in order to maintain high levels of selection pressure throughout somatic embryo formation and regeneration. Further evaluation based on phenotypic expression of the introduced gene will be carried out by spraying glasshouse-grown plants with the Buster herbicide.

The cost of producing a transgenic plant via indirect and direct somatic embryogenesis was calculated based on media components and labour costs. Explants from the direct route required fewer subcultures on to fresh media and more plants were regenerated. This is reflected in the lower cost per plant (R52), four times less than required to produce a transgenic plant via the indirect route (Table 1).

Conclusions

Although higher proportions of nontransformed plants regenerate via the direct somatic embryogenic route when compared to the conventional indirect route, the reduced cost per plant and higher throughput indicate that direct somatic embryogenesis has the potential to increase the overall efficiency of a sugarcane transformation programme.

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