COMPARISON OF SELECTED COMMERCIAL SUGARCANE VARIETIES FOR USE IN A STABLE TRANSFORMATION PROGRAMME

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Abstract

Ten commercial sugarcane varieties, N12, N14, N19, N24, N25, N26, N27, 75E247, 84E1334 and NCo376 were tested for suitability for transformation in terms of their embryogenic callus production and transient expression levels. Based on an increase in callus mass over time, varieties N12, N19, N27, NCo376 and 84E1334 outperformed other varieties on Murashige and Skoog medium containing 2,4-dichlorophenoxyacetic acid (3 mg/L). Using microprojectile bombardment and a plasmid which carries the maize anthocyanin gene, transient expression levels of varieties were determined by counting the number of red foci per cm^2 callus material 48 hours after delivery. Varieties NCo376, N12, N27 and N19 showed high transient expression levels of between 1 000 and 1 450 red foci per cm^2 callus material. Regeneration regimes for varieties differed and these are discussed. Three varieties, NCo376, N12 and N19, were selected for further stable transformation studies on the basis of promising performance.

Introduction

Genetic engineering can be used to modify crop species in a directed manner, producing useful variants in shorter time periods than traditional plant breeding (McElory and Bretell, 1994). Three basic requirements must be met to facilitate the use of tissue culture techniques in plant breeding and genetic engineering: callus growth after initiation, callus differentiation into plantlets and stable insertion, integration and expression of characterised genes into the host genome (Vasil, 1987).

Four distinct types of callus have been observed in sugarcane (Taylor *et al.*, 1992) with embryogenic callus being the most suitable for transformation (Bower and Birch, 1992; Snyman *et al.*, 1996). Although one of the most important factors affecting callusing frequency in sugarcane appears to be explant age, there is an interaction between culture medium composition and genotype (Lui, 1993). Plant regeneration may proceed via organogenesis, requiring separate culture media for shoot and root formation, or embryogenesis, where roots and shoots develop simultaneously (Ho and Vasil, 1983). Regeneration rates and culture conditions vary greatly among cultivars of a single plant species (Fisk and Dandekar, 1993). Transformation of embryogenic sugarcane has been achieved using microprojectile bombardment in only a few varieties (Bower and Birch, 1992). The most critical parameter appears to be the ability of different varieties to produce embryogenic callus (Bower and Birch, 1992).

NCo310 is the only transformable South African variety reported in literature and is thus used as a 'standard' (Gallo-Meagher and Irvine, 1993; Snyman *et al.*, 1996). Previous work has determined suitable bombardment parameters for transient expression using this variety, but NCo310 is no longer a commercial variety in the sugar industry. Consequently, other varieties suitable for use in a stable transformation programme are required.

The work reported in this paper was designed to investigate the potential of ten commercial varieties to form embryogenic callus, to transiently express inserted gene sequences and to regenerate into plantlets. Stable transformation was further investigated in three of the ten varieties.

Materials and methods

Explant material and in vitro callus initiation and maintenance

Callus was initiated from leaf roll tissue of mature, field-grown sugarcane varieties N12, N14, N19, N24, N25, N26, N27, 75E247, 84E1334 and NCo376 and maintained on standard Murashige and Skoog (MS) medium containing the growth hormone 2,4-dichlorophenoxyacetic acid (2,4-D) (3 mg/L). All protocols were as described previously for use with NCo310 (Snyman *et al.*, 1996). In some cases, 2,4-D concentrations were alternated on a weekly basis between 1 and 3 mg/L in an attempt to increase callus production.

Plasmid constructs

Four different plasmids were used. Plasmid p1 contains the maize anthocyanin gene that initiates anthocyanin production, while plasmid p2 contains the neomycin phosphotransferase II (*nptII*) gene that confers resistance to the antibiotic geneticin. Plasmid p3 contains the eldana (*Eldana saccharina* Walker [Lepidoptera: Pyralidae]) resistance gene (*ERI*) and plasmid p4 contains both the *nptII* and *ERI* genes.

DNA precipitation on to tungsten particles and microprojectile bombardment

Biologically active DNA was coated on to tungsten particles and bombarded into embryogenic sugarcane callus using protocols previously described (Snyman *et al.*, 1996).

Monitoring transient expression

Transient expression of the anthocyanin gene was assessed 48 hours after bombardment by enumeration of red foci (Snyman *et al.*, 1996).

Selection of stably transformed plantlets

Callus cobombarded with plasmids p2 and p3 or bombarded with plasmid p4 was placed onto callus induction medium and incubated in the dark at 26°C for one week. Thereafter, callus was placed on selection medium (MS medium containing 15 mg/L geneticin and 1 mg/L or 3 mg/L 2,4-D) in the dark for two weeks with weekly subcultures. The embryogenic structure of the callus was maintained by visually assessing the status of the callus for reversion to yellow, non-embryogenic forms. Reversion to yellow callus was reversed by cycling on different concentrations of 2,4-D. After two weeks, callus was placed in the light on medium containing 15 mg/L geneticin without hormone. Every two weeks callus was subcultured until shoot and root formation had occurred. At this point, plantlets were placed on medium without selection pressure or hormone and allowed to grow before being potted.

Results and discussion

Variation in embryogenic callus production

All ten commercial sugarcane varieties were assessed on their ability to produce embryogenic callus on standard medium. Nine weeks after initiation, embryogenic callus mass from each variety was compared to that produced by NCo310. Varieties N14 and N26 produced little or no embryogenic callus and were discarded. The mass of embryogenic callus produced by N12, N19, N27, NCo376, 84E1344 was twice that produced by N24 and N25 and compared favourably to that produced by NCo310. This variation in the growth response of different genotypes has been reported by other authors who have been able to produce embryogenic callus from a selection of sugarcane varieties by manipulating the culture medium components (Ho and Vasil, 1983; Liu, 1993). Further studies could therefore investigate the effect of different media and 2,4-D concentrations on callus production in potentially important varieties.

Three varieties, N12, N19 and NCo376, were subjected to alternating 1 and 3 mg/L 2,4-D concentrations. This did not produce significantly different callus quantity, but the overall embryogenic nature of the callus appeared to be improved. These results support those of Chen *et al.* (1988) who found that alternating low and high 2,4-D concentrations maintained the ability of sugarcane callus to form somatic embyros.

Variations in transient gene expression

Genotypic differences were apparent when transient expression levels were compared. Highest transient expression levels (2000 to 2200 red foci per cm^2) were shown in varieties N12, N19, N27 and NCo376, moderate levels (approx. 1800 red foci per cm^2) in 84E1334 and 75E247 and low levels (approx. 1400 red foci per cm^2) in N24 and N25. These results correlate with those observed for embryogenic callus production, and may be attributed to higher metabolic activity associated with embryogenic cells (Vasil *et al.*, 1991). Low transient expression efficiency may be ascribed to inadequate penetration of cells by tungsten particles (Kemper *et al.*, 1996). Future attempts to increase transient expression could include varying helium pressures above those used in this study.

Stable transformation and regeneration under selection

Three of the varieties (N12, N19 and NCo376) producing large amounts of embryogenic callus and high transient expression levels were chosen for stable transformation work. These varieties were cobombarded with plasmids p2 and p3 or bombarded with plasmid p4. Cobombarded callus did not regenerate on selection medium containing 15 mg/L geneticin, but callus transformed with plasmid four yielded a few plantlets (1, 5 and 12 plants for N12, N19 and NCo376, respectively). As regeneration of all varieties was poor, future work to determine optimal medium composition for regeneration of each variety appears necessary.

Geneticin has been successfully used for the selection of transformed cells in sugarcane (Bower and Birch, 1992). In this study, however, very few plantlets were regenerated under geneticin selection. There are two possible explanations: either the regeneration medium used was not optimal for the varieties or the selection protocol was too stringent. Preliminary work has shown that each variety tested has different geneticin susceptibilities and implies that the levels of antibiotic required have to be determined for each variety transformed.

Conclusions

Six varieties, N12, N19, N27, 84E1334, 75E247 and NCo376, performed well in terms of their embryogenic callus production and transformation efficiency as their potential for callus production and transformation compared favourably to that obtained with NCo310. These varieties could be used successfully for stable transformation providing that future studies concentrate on establishment of suitable selection and regeneration protocols for each variety.

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