NEW DEVELOPMENTS IN THE PRODUCTION OF HERBICIDE-RESISTANT SUGARCANE

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Abstract

In a previously reported model study, sugarcane was genetically engineered with the *pat* gene conferring resistance to the herbicide Buster®. The sugarcane cultivar used in that work was NCo310, which is no longer grown commercially but responds well to tissue culture and transformation. The field performance of the herbicide resistant sugarcane was assessed and stable transgene expression observed over several ratoons with repeated herbicide application. However, the cost of the Buster® herbicide is particularly high. The use of a cheaper herbicide known to be as effective as Buster® could increase the returns per Rand spent on weed control by up to six times. More recently, therefore, a gene conferring resistance to a cheaper herbicide has been introduced routinely into commercial cultivars N12 and N19, which constituted 32% and 16% respectively of cane harvested in South Africa in 2000-01. Gene delivery by microprojectile bombardment was accomplished using five plasmid constructs, each containing the same herbicide resistance gene and the antibiotic selectable marker gene *nptII*, but differing in promoter characteristics. Plantlets were regenerated via either direct or indirect somatic embryogenesis and selection carried out on medium containing the antibiotic geneticin. Putatively transformed plants were subjected to herbicide spraying in the glasshouse where to date 52% have survived a sub-lethal dose, which severely damaged control plants. Results have suggested that N19 is more amenable to transformation and regeneration than N12. In addition, it has been shown that the promoters chosen to drive the gene of interest and selection gene are of great importance. In this study CaMV 35S promoter derivatives proved to be effective in the genetic environment of sugarcane.

Rationale

Sugarcane has been successfully genetically engineered for resistance to the herbicide Buster® (Gallo-Meagher and Irvine, 1996; Snyman *et al*., 1998), although in those previously reported studies the cultivar transformed was NCo310, which is no longer grown commercially. Subsequent field trials showed stable inheritance and expression of the gene through multiple ratoons. However, the cost of using Buster® in South Africa is prohibitively expensive. In experimentally determining the economic advantage of a herbicide resistant sugarcane crop (Leibbrandt and Snyman, 2001), it was reported that the use of a cheaper herbicide could significantly increase the returns per Rand spent on weed control. In addition, the gene conferring resistance to the cheaper herbicide could be introduced into commercial cultivars, e.g. N12 and N19, which constitute 32% and 16% respectively of cane harvested annually in South Africa ([1]KJ Nuss, personal communication).

In the study reported here, a suitable herbicide resistance gene was introduced into two sugarcane cultivars, N12 and N19, via microprojectile bombardment of somatic embryos generated by both direct and indirect pathways of morphogenesis. In addition, the transformation efficiencies of five vector constructs differing in promoter usage were compared. Successful transformation was measured by phenotypic expression of herbicide resistance following glasshouse spraying trials. Preliminary development of molecular techniques for laboratory detection of the introduced gene in the plant genome at an early stage of plantlet development was also undertaken.

***In vitro* culture and transformation by microprojectile bombardment**

Gene constructs were introduced by microprojectile bombardment into somatic embryos of cultivars N12 and N19 developed in culture by one of two routes: (1) indirectly via callus (Snyman *et al*., 1996) or (2) directly on immature leaf roll discs (Snyman *et al*., 2000). Five constructs were used, each one a circular plasmid containing both the herbicide resistance gene (*HR*) and the antibiotic selectable marker gene (*nptII).* Expression of the latter allows selection of transformed cells on medium containing the antibiotic geneticin. In each of the five constructs, different combinations of promoters potentially suitable for monocotyledonous plant expression were used to drive expression of the two genes. Each construct was delivered independently.

Induction and maintenance of embryogenic callus was in the dark on MS (Murashige and Skoog, 1962) basal salt and vitamin medium containing sucrose (30 g/l), casein hydrolysate (1 g/l), 2,4-D (3 mg/l), agargel (Sigma; 5 g/l), pH 5.8. Calli were subcultured at two week intervals and bombarded (Finer *et al*., 1992; Snyman *et al*., 1996) after 12 weeks in culture. Leaf discs were cultured on a similar medium, except that 2,4-D levels were reduced to 0.3 mg/l and explants were bombarded after 10-14 days. Bombarded calli and leaf discs were placed on selection medium (MS plus geneticin (15 mg/l)) four days after bombardment, and were subcultured fortnightly. Developing somatic embryos, ready for transfer to regeneration medium (MS plus 0.5 mg/l kinetin, without 2,4-D) in the light, were observed on the callus mass after 12 weeks or formed directly on the leaf discs after eight weeks. Once plantlets were approximately 10 cm tall, they were hardened off in the glasshouse.

Phenotypic analysis of transgenic plants by glasshouse spraying

Phenotypic assessment of the putatively transgenic plantlets was carried out in the glasshouse 4-6 months after the hardening off process. Plants were sprayed to full cover with a hydraulically operated knapsack at a sub-lethal dose of 2-4 l/ha herbicide. Plants were assessed for phytotoxic damage once a week for three weeks. Sprayed plants exhibited varying degrees of damage two weeks after herbicide application. This may be related to promoter, gene copy number or positional effects (Birch *et al*., 1996). Of 173 plants that were spray-tested (N12 and N19; all 5 construct variants), 90 plants (52%) survived a dose of 2 l/ha herbicide and of these, 22 (12.7%) survived an accumulative dose of 6 l/ha herbicide.

Significance of cultivar and promoter

Using a subset of plants regenerated from embryogenic callus, the effect on regeneration efficiency of the different plasmid constructs containing different promoter elements is shown in Table 1. In both N12 and N19, the highest numbers of regenerated plants and highest regeneration efficiencies (measured as number of plants per bombardment), were obtained using plasmid pHR5. This plasmid makes use of synthetic variants of the cauliflower mosaic virus (CaMV) 35S promoter. Since, in the single plasmid pHR5, there are two somewhat different CaMV 35S promoter variants driving the antibiotic selection and herbicide resistance genes respectively, it is not possible to measure whether the results are due more to selection efficiency than strong resistance gene expression, or vice-versa. As the two promoters in pHR5 have basic similarities, the likelihood is that both processes are efficient in sugarcane. Success may be partly ascribed to the introduction into the promoter structures of enhancer, leader sequence and intron elements, which have been shown to stabilise transgene expression (Vain *et al*., 1996).

In a comparison of the number of transgenic plants from the five plasmid constructs in the two cultivars, regardless of the type of embryogenic material used (Table 2) the superiority of pHR5 is confirmed. It can be seen that 51 (100%) N19 plants were resistant to the herbicide, whereas in N12 only 16 of the 38 (42%) sprayed plants displayed the herbicide resistant phenotype. Overall, Tables 1 and 2 indicate that genotypic differences in the parent material also play a role in determining efficiency and that N19 is more amenable to transformation and regeneration than N12.

Molecular analyses: PCR and dot blot

With a view to devising techniques of rapid molecular analysis to detect the presence of the transgene in sugarcane plantlets prior to hardening off and glasshouse transfer, a subset of plants were PCR analysed for the presence of the *HR* and *nptII* genes. Although the *nptII* gene amplicon was obtained for all the plants tested, indicating that transformation had occurred, none of those plants tested positive for the *HR* gene fragment (results not shown), despite successful amplification of the *HR* gene in the plasmid vector. These results suggest that, although the primer design is appropriate, further optimisation will be necessary to detect the *HR* gene in a complex genomic environment, especially if it is present in low copy number.

Conclusions

The herbicide resistance gene, *HR*, selected to provide an alternative to Buster® resistance was successfully introduced into two sugarcane cultivars, N12 and N19, via microprojectile bombardment. The cultivar N19 appears to be more amenable to transformation and regeneration than N12, demonstrating that parent genotype plays an important role in the genetic engineering process. The promoters chosen to drive the gene of interest and selection gene are also of great importance. In this study the vector pHR5 containing CaMV 35S promoter derivatives proved to be effective in the genetic environment of sugarcane. The vector pHR5 will therefore be used extensively in future transformation protocols. Future molecular work will include Southern hybridisation analysis on a selected subsample of plants to investigate the relevance of copy number and integration patterns. Glasshouse herbicide spraying trials have been shown to be most reliable in establishing transgenic plant output, although optimisation of *HR* gene PCR should be pursued, as this would allow earlier diagnosis of the presence of the transgene and non-transgenic plants could be eliminated at the laboratory stage.

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Table 1. Comparative regeneration efficiencies obtained using cultivars N12 and N19 and five vector constructs differing in the combination of promoters driving the herbicide resistance (*HR*) and *nptII* genes. Embryogenic callus was the target material and the DNA was delivered on gold particles.

Plasmid construct	Number bombardments		Number regenerated plants		Regeneration efficiency (number plants per bombardment)	
	N12	N19	N12	N19	N12	N19
pHR1	50	70	0	49	0	0.7± 0.08
pHR2	120	133	18	13	0.15± 0.01	0.1± 0.01
pHR3	64	118	61	0	0.95± 0.12	0
pHR4	30	103	0	118	0	1.14± 0.11
pHR5	50	47	78	139	1.56± 0.08	2.96± 0.43

Table 2. Effects of cultivar and promoter combinations on transgenic plant production, regardless of the target material used.

Plasmid construct	No. plants spray-tested		No. of survivors		Transgenic plants (%)	
	N12	N19	N12	N19	N12	N19
pHR1	-	15	-	10	-	67
pHR2	14	8	0	7	0	88
pHR3	18	-	0	-	0	-
pHR4	-	29	-	6	-	21
pHR5	38	51	16	51	42	100