FINAL REPORT to SUGAR RESEARCH AND DEVELOPMENT CORPORATION

Project code:	UQI2S
Project title:	High Efficiency Production of Transgenic Sugarcane Plants
Field of Research:	The Cane Plant **Field Code:** 1
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The University of Queensland provided the salary of the project supervisor, and costs of laboratory equipment and specialised facilities necessary for the project (estimated cost $30,000 p.a.).

Project Objectives:

The aim of this project was to increase the frequency of production of transgenic sugarcane plants following particle bombardment, for efficient testing of new genes in sugarcane improvement projects. Preliminary indications were that an increase of up to 10-fold might be achievable.

Non-Technical Summary

The efficiency of gene transfer into embryogenic callus of sugarcane has been increased tenfold by optimisation of particle bombardment conditions, and there is a corresponding increase in stable transformation frequencies. The method routinely yields approximately 2 independent transgenic plants per cm^2 of bombarded embryogenic callus for sugarcane varieties amenable to tissue culture. Genes coprecipitated on separate plasmids are cotransformed at high efficiency, which will facilitate introduction of agronomic genes. Materials needed for recovery of transgenic plants can be halved through improved selection protocols, allowing the recovery of hundreds of independent transformed plant lines. The improved method is now in use in BSES and CSIRO as well as UQ laboratories Since the completion of this project, the transformation system has been shown to be effective on a range of major commercial varieties, and over 70 lines from seven cultivars are currently in field trials.

Technical Summary

Transient expression of the maize anthocyanin regulatory elements, R and C1, was used to optimise parameters affecting efficiency of delivery of DNA into sugarcane embryogenic callus cells by microprojectile bombardment. Use of a high pressure helium pulse proved effective in accelerating microprojectiles with minimum tissue damage. In conjunction with osmoticum treatment of target tissues and increased shot volumes, 5000-8000 transiently expressing cells per 3.5 cm diameter target were routinely observed. An average of 0.34% of these developed into stably transformed anthocyanin pigmented proembryoid structures which subsequently regenerated into plantlets. All anthocyanin pigmented plants died while less than 1.5 cm high. We compared selection of bombarded callus on phosphinothricin or geneticin with selection based on selective subculture of callus portions showing luciferase activity. Phosphinothricin was ineffective in selecting transformed callus of cv Q117. Selection based on visualisation of luciferase activity enabled recovery of 1.4 ±0.5 *luc* expressing plant lines per bombardment, compared to 19.8 ±3.7 independent transformants recovered using an optimised selection regime on geneticin. Coexpression rates for a coprecipitated *luc* gene in callus lines selected for geneticin resistance ranged from 67-79%.

Background & Introductory Technical Information

This project built on the successful outcome of SRDC project UQ1S, which developed a system for production of transgenic sugarcane plants following gene transfer on high velocity DNA-coated microprojectiles. The gene transfer efficiency for a skilled operator working with good quality embryogenic callus was 0.1 to 0.2 transformants cm^{-2}, or about one transgenic sugarcane plant per 3 cm diameter target area bombarded (Bower and Birch 1992).

This is sufficient for the transfer of individual known useful genes into sugarcane with the objective of selecting commercially useful transformants. However, when projects require the introduction of multiple genes into a range of sugarcane varieties, a large amount of tissue cultured material must be handled to produce sufficient high quality target tissue and select the desired number of transformants. This is labour intensive and time consuming, and therefore expensive. At the current gene transfer efficiency, this rapidly becomes the logistic factor which limits what can be achieved in practice in projects requiring gene transfer into sugarcane.

A good example of a problem area is genetic manipulation for increased sucrose accumulation, where the best current approach is to experimentally alter levels of expression of a range of key enzymes or transporters involved in sucrose metabolism and storage. A high transformation frequency is a key requirement for such projects, which need to generate a substantial range of transgenic plants to test hypotheses or establish *which* genes and expression patterns are useful, rather than a few plants for immediate commercial evaluation. Increased transformation frequency was recognised as a high research priority at the recent SRDC sucrose accumulation workshop, although the means to achieve such an increase were not identified (Birch, 1992).

As part of the work in UQ1S we made preliminary tests of promising new transformation techniques or improved protocols described by other research groups. In our hands, most such techniques have been completely ineffective for sugarcane, or inferior to our current protocols. However, a 10-fold increase in transient expression frequency in bombarded embryogenic callus was achieved in one of these preliminary experiments near the end of UQ1S, using modified bombardment conditions (particle size, DNA concentration, precipitation method and acceleration mechanism all differed from our routine protocol).

Research Methodology

Overview

During the 7 month project UQ12S we completed a series of experiments with the modified protocol in parallel with the previous routine protocol; using embryogenic callus lines, reporter genes and assays established in UQ1S and described in detail elsewhere (Bower & Birch 1992).

Target Tissues

Embryogenic callus cultures of sugarcane cultivar Q117 were established as described by Franks and Birch (1991). Callus was bombarded as soon as possible after initiation, usually requiring 10-12 wk to form and multiply to usable quantities. Segments of embryogenic callus of 4-8 mm diameter were placed on MSC_3 medium (Heinz and Mee 1969), supplemented with 0.2 M mannitol and 0.2 M sorbitol as an osmotic treatment (Vain *et al.* 1993) for 4 hr prior to bombardment and 4 hr post-bombardment, unless stated otherwise. A circular target area was used, varying between 2-3.5 cm diameter in different experiments.

Microprojectile Bombardment Conditions

A gas control solenoid and syringe filter holder, as described by Finer *et al.* (1992) were arranged to replace the barrel and stop plate in the apparatus described previously (Franks and Birch 1991). When performing coprecipitation of two plasmids 2.5 ug of each was premixed prior to addition to the precipitation mix. DNA was precipitated onto tungsten particles as described by Finer *et al.* (1992) except that 50 ul of supernatant was removed after precipitation for 10 min on ice. The tungsten was resuspended and, unless stated otherwise, a 4 ul aliquot placed on the support grid. A nylon mesh screen (500 um aperture, 50% open area, Swiss Screens Pty Ltd) was placed 7.5 cm above the target tissues, which were positioned 16 cm below the particle outlet. The vacuum chamber was evacuated to 28" Hg (6.5 kPa absolute pressure) and the microprojectiles accelerated by a 50 msec helium pulse, at 1200 kPa unless otherwise stated. The vacuum was released immediately after bombardment.

Culture Conditions

After bombardment and incubation on medium containing osmoticum callus was transferred to MSC_3 for 4 d, unless stated, then placed on MSC_3, with or without selective agents, and incubated at 28^{0}C in the dark. Callus was subcultured every 14 d. Regeneration of plants was induced by transfer to medium (MSC) lacking 2,4-dichlorophenoxy acetic acid, plus selective agents where required. Regenerating callus and plantlets were maintained at 28^{0}C under fluorescent lights.

Reporter Gene Expression Assays

Transient expression assays were performed 48 hr after bombardment. GUS histochemical assays entailed immersion of callus in GUS assay buffer (Klein *et al.* 1988) overnight at 35^{0}C, after which blue cells were counted. ANT assays consisted of simply counting red cells or callus segments under a stereo microscope. LUC assays were performed by immersion of callus or plants for 10 min in 0.4 mM luciferin. Light emission was imaged using a liquid nitrogen cooled CCD camera and AT1 software (Wright Instruments Ltd Enfield UK), as described by Mudge *et al.* (1995).

Selection Procedures

Three methods were tested for selection of transformed tissues. Visualisation of luciferase activity at 20 d post bombardment was used to identify callus portions which were maintained on MSC_3 until 56 d post bombardment when they were placed on regeneration medium. Plantlets were tested for luciferase activity. This method was compared with selection on media containing either geneticin (Sigma) or phosphinothricin (Hoechst Pty Ltd). Appropriate concentrations of these selective agents were determined for both callus and plantlets. The comparison of LUC based selection with selection on geneticin was performed by coprecipitation of the *luc* and *aph*A genes and division of bombarded callus into either treatment to negate differences in precipitation efficiency. Bombarded callus was divided into portions about 5 mm diameter for culture on selective medium. Numbers of transformants were assessed by the ability of callus to regenerate plants capable of rooting on full selection. We have confirmed the reliability of this criterion by Southern analysis of 75 independent transformants, all of which contained the expected sequences.

Plant Expression Vectors

The plasmid pDP687 (kindly supplied by Pioneer Hybrid Seeds) was used to confer anthocyanin (ANT) expression. It consists of the maize anthocyanin R and C1 regulatory elements each preceded by a maize alcohol dehydrogenase intron 1 at the 5' end, followed by a *Pin*II terminator sequence at the 3' end, and each driven by a dual cauliflower mosaic virus 35S (CaMV35S) promoter sequence. Plasmids pOsaPO and pOsaGO (kindly supplied by CSIRO Plant Industry, Canberra) conferred phosphinothricin resistance (*bar*) and GUS expression (*uid*A) respectively, driven by an artificial promoter consisting of four tandem octopine synthase (OCS) enhancer elements, and a minimal CaMV35S promoter core region. Both genes were preceded by tobacco mosaic virus and *Ac* untranslated leader sequences and used an OCS terminator sequence. The plasmid pOsaLO, conferring luciferase activity, was constructed by a *Hin*dIII/ *Sac*II excision of the firefly *luc* gene from pGEMLUC (Promega), blunting of this fragment using T_7 polymerase, followed by its ligation into pOsaPO from which the *bar* gene had been excised by *Sma*I.

pAHC18 (Bruce *et al.* 1989) was also used to confer luciferase activity driven by a maize ubiquitin promoter. pEmuKN (Last *et al.* 1991) was used to confer resistance to geneticin.

Detailed Results

High frequency gene transfer

To optimise the gene transfer procedure, we measured the effects of a range of parameters on numbers of transiently expressing cells in embryogenic callus, in conjunction with qualitative assessment of degree of tissue damage. The reporter genes *uid*A (GUS), firefly *luc* (LUC) and the maize anthocyanin regulatory elements R and C1 (ANT) were tested as reporters of transient expression. LUC producing cells often appeared as confluent patches of glowing tissue, making quantification of numbers of expressing cells difficult, whereas GUS and ANT gave excellent spatial resolution. To estimate the relative sensitivities of the GUS and ANT reporter gene systems, embryogenic callus was bombarded with pOsaGO and pDP687 respectively. Counts of transiently expressing cells showed 875 ± 49 GUS producing cells compared with 2222 ± 137 ANT producing cells indicating that the ANT system is more sensitive. In addition the ANT system does not require expensive substrates as used in GUS and LUC assays. ANT producing cells are easily visible and we have observed no background problems unless callus is exposed to bright light for 24-48 hr. Anthocyanin pigment becomes visible in expressing cells as early as 8 hr after bombardment, steadily intensifies until about 48 hr, then remains visible for 2-3 wk. The ANT system was used for all transient expression experiments described here.

A helium pulse was applied at increasing pressures as a method of microprojectile acceleration (Finer et al. 1992). Highest transient expression rates occurred at 1200 kPa (Fig 1), the highest pressure tested. Subsequent experiments showed a continuing increase to 1500 kPa (data not shown), but to minimise tissue damage 1200 kPa was used in the experiments described here.

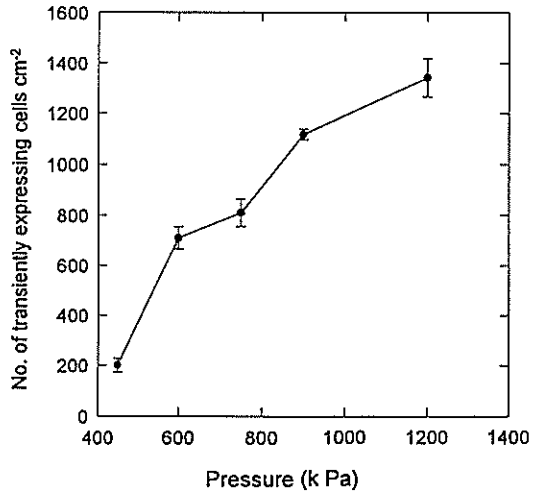

Figure 1. Effect of accelerating helium pressure on frequency of anthocyanin reporter gene expression in bombarded embryogenic callus.

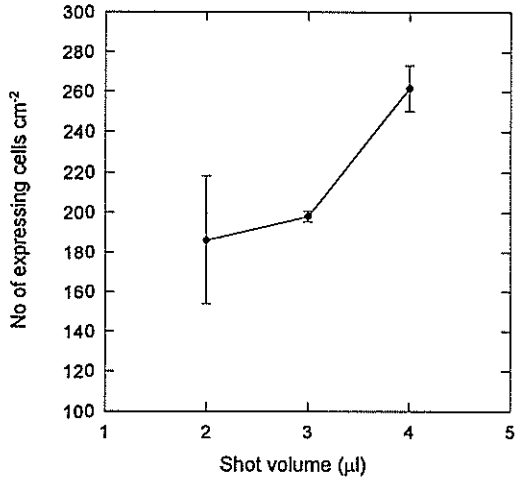

Figure 2. Effect of volume of accelerated particle suspension on frequency of anthocyanin reporter gene expression in bombarded embryogenic callus.

Increasing the volume of microprojectile suspension loaded onto the carrier mesh resulted in increasing numbers of transiently expressing cells (Fig 2), with no apparent increase in tissue damage. Volumes larger than 4 ul could not be conveniently loaded onto the carrier mesh.

Osmoticum treatments of target tissue resulted in a large increase in numbers of transiently expressing cells. Four hr incubation on osmoticum medium prior to bombardment was sufficient to maximise transient expression numbers with longer periods slightly decreasing efficiencies (Fig 3). Similarly 4 hr post-bombardment incubation on osmoticum gave the best results (Fig 3). Incubation of callus on osmoticum enabled transient expression in a softer less compact callus type which we had previously found incapable of expression under our bombardment conditions.

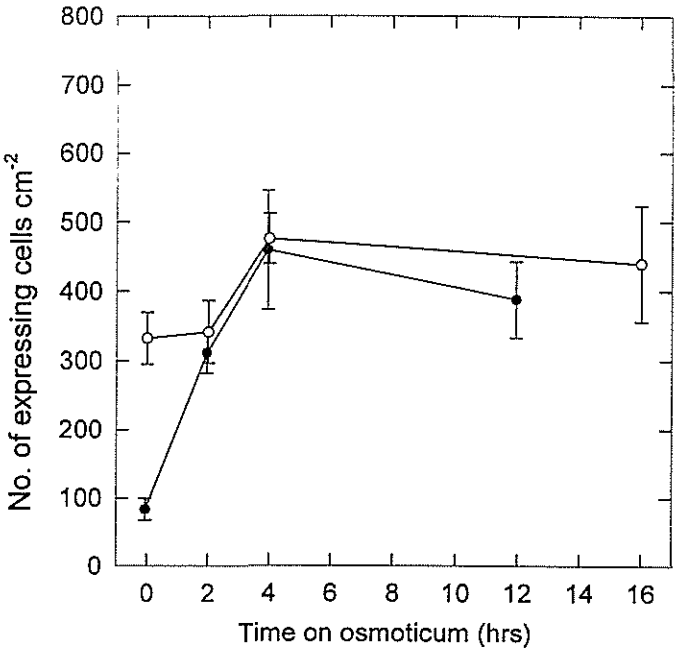

Figure 3. Effect of osmotic treatment before (closed circles) and after (open circles) bombardment, on frequency of anthocyanin reporter gene expression in embryogenic callus.

Substitution of tungsten with gold particles did not significantly alter transient expression numbers (tungsten 1982±280; gold 2090±444, mean±SE of 5 reps).

The modified apparatus and conditions described above increase transient expression frequencies approximately 10-fold compared to those produced using our initial system (Bower and Birch, 1992). Bombardment of embryogenic callus routinely yields 160-260 positive cells / cm^2 in transient GUS assays, or 400-650 positive cells /cm^2 using the anthocyanin reporter system.

Selection and Regeneration of Transformed Plants

Improved selection regimen for antibiotic resistance

Stepped selection on increasing concentrations of geneticin is effective for recovery of transgenic sugarcane plants (Bower and Birch, 1992), but callus proliferation before transfer to escape-free selection can present logistical problems for large scale transformation work. Several methods involving more rapid transition to escape-free selection substantially reduced the amount of callus material to be handled, without reducing the number of transgenic plant lines recovered (Table 1). For cultivar Q117, we now routinely allow 4 days recovery on MSC_3 medium after bombardment, followed by four to six subcultures of 2 weeks duration on medium containing 45 mg l^{-1} before regeneration. This typically yields 15 to 20 transformed plant lines per 3.5 cm diameter target area of embryogenic callus.

Table 1. Effect of three selection regimens on efficiency of selection of transformed sugarcane plants on media containing geneticin.

Selection stage		Method 1	Method 2	Method 3
Microprojectile bombardment		+	+	+
Callus growth without selection (MSC_3 medium)		2 weeks	-	2 weeks
Callus growth with sublethal selection	15mg l^{-1}	2x2 weeks	2 weeks	-
Callus growth with sublethal selection	25mg l^{-1}	-	2 weeks	-
Callus growth with sublethal selection	35mg l^{-1}	2 weeks	-	-
Callus growth under escape-free selection	45mg l^{-1}	2 weeks	3x2 weeks	4x2 weeks
Plant regeneration on escape-free selection	45mg l^{-1}	+	+	+
Total time from bombardment to regeneration		10 weeks	10 weeks	10 weeks
Transgenic plants per bombardment (2.8 cm diam.)		3.6 ± 0.9	4.8 ± 1.8	4.0 ± 0.8
Plates subcultured per bombardment		24.4 ± 1.7	14.8 ± 1.4	11.8 ± 1.5

Attempted selection for herbicide resistance

In our hands, selection for phosphinothricin resistance has not been successful for recovery of transgenic sugarcane plants. We find a large variation between experiments in the phosphinothricin concentration required to kill untransformed callus of the same sugarcane cultivar. Phosphinothricin sensitivity also appears to vary with callus type and developmental stage. For cultivar Q117, the escape free selection level for rapidly growing embryogenic callus varied from 10 - 80 mg l^{-1}. Non-embryogenic callus appeared more resistant, and overgrew embryogenic callus. Regenerated plantlets were much more sensitive, being rapidly and consistently killed on media containing 2 mg l^{-1} phosphinothricin.

Selective subculturing based on luciferase or anthocyanin expression

We also investigated selective subculturing of callus expressing luciferase or anthocyanin reporter genes as an alternative to the use of antibiotic selection for recovery of transgenic plants. Most pigmented cells detected 48 hr after bombardment with the anthocyanin construct retained their red pigmentation for up to three weeks without dividing. An average of 0.35% continued to divide and multiply, forming red pro-embryoid structures which developed into plantlets on regeneration medium. However, these plantlets generally exhibited unusual morphology, such as thick and slightly twisted leaves, and no red plantlet survived to a height greater than 1.5 cm. Darker coloured plantlets died sooner. The ANT system clearly revealed chimaeric transformants if regeneration was allowed soon after bombardment, but plants regenerated after the usual time for selection were uniformly transformed.

For the luciferase system, callus regions expressing the introduced gene 20 days after bombardment were detected under an ultra-low light camera, and selectively transferred for further growth on MSC_3 medium until transfer to regeneration medium 56 days following bombardment. Massive proliferation of callus occurred in the absence of selective agents, and callus tended to assume a nonembryogenic state after immersion in luciferase assay buffer. One to two luciferase expressing plantlets were recovered per bombardment, but the large excess of untransformed material made this approach impractical for routine use.

Coexpression rates

Assays for luciferase activity were performed on calli co-bombarded with *aph*A (NptII) and *luc* genes on separate plasmids, and selected for geneticin resistance (*AphA* expression). In separate experiments cobombarded with pEmuGN and pOsaLO or pAHC18, the frequencies of luciferase expression in geneticin resistant calli were 67% and 79% respectively.

Discussion

Optimisation of parameters affecting delivery of DNA into sugarcane embryogenic callus cells and their subsequent survival were assisted by the sensitive and convenient ANT marker gene system. This system has previously been used successfully in various cell types in a range of cereal species (see Bowen, 1992). The sensitive and inexpensive assay for transient expression and subsequent development of stably transformed sectors, made it possible to follow the growth of single transformed sugarcane cells into transformed plantlets. This was of interest for better understanding of patterns of growth and regeneration of embryogenic callus, as a basis for decisions about the timing of selection and regeneration treatments.

However, our results suggest that use of R and C1 transcriptional activators as marker genes for stable transformation is limited, due to deleterious effects on development resulting in death of plantlets. This could be a direct consequence of excessive anthocyanin accumulation, or due to the effects of high levels of the transactivating factors R and C1 on transcriptional control of other genes.

DNA coated particles accelerated in a helium gas stream were delivered with high efficiency into sugarcane embryogenic callus cells. Optimal helium pressure was approximately double that used by Finer *et al.* (1992) for delivery of DNA into cowpea leaves and maize embryogenic suspension cells. Incubation of target tissues on medium containing an osmoticum to minimise damage to cells resulting from penetration by tungsten particles greatly increased numbers of transiently expressing cells, as reported for other plant cells (Ye *et al.*1990, Perl *et al.*1992, Vain *et al.*1993, Leduc *et al.* 1994). We found similar increases in transient (X 5.5) and stable (X 4.8) transformation rates due to osmotic treatment. Vain *et al.* (1993) reported a 2.7 times increase in transient *uid*A expression and a 6.8 times increase in stable transformants. It has been observed that damaged cells can accumulate the GUS enzyme but not anthocyanin pigment, due to a requirement for an intact vacuole and coordinate expression of many genes for the latter process (Bowen, 1992). Thus similar increases in transient and stable transformation rates for ANT might be expected if the effect of the osmoticum is to protect cells from damage during bombardment. The rate of 0.35% conversion from transient to stable transformation is lower than most estimates using the GUS system (eg 1.9%, Klein *et al.* 1988; 0.8%, Hunold *et al.* 1994). This could be a result of the higher sensitivity of detection of transient expression from the ANT system, if stable transformation is favoured by higher DNA loads per cell, or it could result from higher attrition

of cells due to adverse effects of overexpression of R and C1 transcriptional factors.

It is possible to recover transgenic sugarcane plants without antibiotic or herbicide selection, based on *luc* expression, as also recently achieved for *Dendrobium* (Chia *et al.* 1994). However, our comparison of selection for LUC versus NPTII activity indicates that selective subculture for LUC activity is 10- to 15-fold less efficient than selection for geneticin resistance. Transformed plants were recovered from less than 5% of cell clusters expressing the *luc* gene 20 d after bombardment, apparently due to the rapid proliferation of untransformed sugarcane callus tissue in the absence of antibiotic selection. However, the *luc* gene is extremely useful in allowing nondestructive assays of gene expression in callus and plants, for purposes such as promoter analysis or tests of the stability of transgene expression.

We found selection on phosphinothricin problematic, due to extreme variation in the concentration required to kill callus, and to a deterioration in embryogenic capacity of callus during selection. In contrast, selection on geneticin is consistent, favours growth of embryogenic over nonembryogenic callus, and has never allowed escapes. Imposition of full selection soon after bombardment has considerably reduced the resources necessary to generate each transformed line, an important consideration in situations requiring introduction of a variety of genetic constructs into several cultivars.

Nehra *et al.* (1994) found selection of transformed somatic wheat embryos on phosphinothricin resulted in 50% escapes and problems in regeneration, while use of geneticin as a selective agent resulted in 20% escapes and normal regeneration efficiency. Phosphinothricin selection has been useful for recovery of transgenic plants in a number of monocot crop species, despite the incidence of escapes from the selection process (Somers *et al.* 1992, Vasil *et al.* 1993, Casas *et al.* 1994). The method gained favour in part because of the tolerance of many monocots to kanamycin (Hauptmann *et al.* 1987). However, unless resistance to phosphinothricin herbicides is a desired outcome of the transformation, selection for resistance to an alternative aminoglycoside antibiotic following introduction of *aphA* may be preferable.

Conclusion

Using a combination of effective microprojectile delivery, osmotic treatments to reduce damage to cells during bombardment and an efficient selection system, we have optimised our transformation system to a degree where 15-20 independent transformants per bombardment are routinely produced in cultivars amenable to embryogenic callus culture, using a 3.5 cm^2 diameter target area. This is a 10-20 fold increase over the initial transformation efficiency. The system is now an effective tool for introduction of agronomically important genes into commercial cultivars, and for investigation of factors affecting yield by manipulating levels of gene expression.

The project objective was successfully achieved, and the optimised protocol is currently being applied for a high transformation frequency in the laboratories working on molecular improvement of Australian sugarcane varieties.

Intellectual Property, Implications and Recommendations

At the end of this project, the transformation efficiency for a skilled operator working with good

quality embryogenic callus is about 2 transgenic sugarcane plants per cm^2 of bombarded callus. This is sufficient for the transfer of useful genes into sugarcane for direct production of improved varieties, and for experiments using genetic manipulation to test hypotheses about processes limiting yield.

The improved efficiency of sugarcane transformation as a result of this project has commercial significance in the sense that it will reduce the cost of sugarcane improvement by genetic engineering, and make possible experiments into processes such as sugar accumulation which were not previously feasible. The improved system is already in use by BSES, CSIRO and UQ groups working on sugarcane molecular improvement.

As discussed in the final report on project UQ1S, patenting of the transformation system is not recommended. The best option for the Australian sugar industry to take commercial advantage of its lead in transformation technology is to move quickly to apply the techniques using available genes, and to support research into novel genes of agronomic importance in sugarcane, which can more readily be protected by patents if desired.

Acknowledgments

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