BUREAU OF SUGAR EXPERIMENT STATIONS

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FINAL REPORT
SRDC PROJECT BS44S
THE PRODUCTION OF TRANSGENIC SUGARCANE PLANTS

by

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SD94005

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1.0 SUMMARY

Transgenic sugarcane plants, which expressed the coat protein of SCMV at a very low level, were produced by microprojectile bombardment of sugarcane meristems. Transgenic plants expressing the luciferase (Luc) and β-glucuronidase (GUS) reporter genes were also produced. A paper describing regeneration of plantlets expressing the reporter gene GUS (Plant Cell Reports 12:343-346) was the first report of the use of sugarcane meristem tissues as a transformation target, and the second report of transgenic sugarcane plants. The level of expression of the coat protein gene in the regenerated plantlets was very low, possibly due to chimaerism, ie mixtures of transformed and non-transformed cells in the same tissue. We have established that sugarcane meristems are a useful target for microprojectile transformation of sugarcane, although more research is necessary before this target can be routinely used for sugarcane genetic engineering.

Several physical and biological factors affected transformation frequency and hence the number of transgenic plantlets produced. The recovery of transformed plantlets was significantly enhanced by the use of cytokinin plant growth regulators in the tissue culture medium. Sugarcane meristem transformation probably can be used to genetically engineer all varieties, although differences between the test varieties in shoot regeneration and transformation frequency were noted. The distance that the nylon macroprojectile, which holds the gold or tungsten microprojectiles, was placed down the gun barrel significantly affected the number of transformed plantlets produced, but did not affect the number of shoots regenerated by the meristem. Dual bombardments did not appear to affect transformation frequency. Different effects were seen in callus tissues transiently expressing the GUS gene after bombardment with plasmid constructs. There was a significant increase in GUS expression after dual bombardments, and there were interactions between callus type, macroprojectile distance and propulsion source. Of particular interest was the finding that gold microprojectiles, which reliably transform meristems, result in very low levels of transformed callus.

2.0 BACKGROUND

Australian sugarcane improvement programs are required to provide faster genetic improvement for additional plant characteristics in order to maintain productivity and ensure the quality of Australian raw sugar. However, increasing the number of criteria for selecting cane varieties usually reduces the rate of genetic improvement for economic traits in conventional breeding programs. Genetic engineering has the potential to increase the productivity of sugarcane improvement programs by the use of novel genes and by efficient manipulation of some plant characteristics.

Production of transgenic plants expressing genes for resistance to sugarcane mosaic virus would increase the productivity of the sugar industry in some mill areas. More importantly, the techniques developed could be applied to other agriculturally useful genes which are being developed by BSES and other institutions.

The SCMV epidemic which affected the Isis mill area of Queensland necessitated the use of less agronomically suitable sugarcane cultivars, as the most suitable commercial varieties at

that time, Q95 and Q137, were both SCMV susceptible. No agronomically suitable SCMV resistant cultivars were apparent in the plant breeding programs for south Queensland at the end of the 1980s, while some agronomically desirable varieties could not be released because of mosaic susceptibility. Resistance to viral infection by expression of viral coat proteins in plant cells has been successfully demonstrated in a number of crops. A construct for expression of the coat protein of SCMV was developed and successfully expressed in sugarcane protoplasts in SRC funded project BS9S `The production of genetic constructs for expression of sugarcane virus coat protein in sugarcane protoplasts'.

Reports from overseas indicated that microprojectile bombardment of plant meristems or callus, and subsequent regeneration of plantlets, is a viable method for the transformation of various plant species, including the monocotyledons *Zea mays* and *Hordeum vulgare*. This technique was investigated to genetically engineer the SCMV synthetic resistance gene into agronomically elite mosaic susceptible varieties of sugarcane.

3.0 OBJECTIVES

1. Establish cell suspension cultures of six cane varieties.

2. Develop efficient protocols for stable incorporation of SCMV coat protein and glyphosate resistance genes in sugarcane protoplasts, using the existing electroporation system.

3. Develop protocols for genetic transformation of sugarcane tissues using a microprojectile gun.

4. Adapt protocols from SRC Project BS3S (Regeneration of sugarcane plants from protoplasts) for preparation of cell lines and plant tissue for transformation with a microprojectile gun.

5. Adapt protocols from SRC Project BS3S for regeneration of sugarcane plants from genetically transformed tissues.

6. Produce sugarcane plants which express genes for the SCMV coat protein and glyphosate resistance.

7. Confirm the relationship between gene expression and plant reaction to SCMV and glyphosate herbicide in glasshouse and field trials (subject to Government regulation).

NB A short version has been used at times, eg in SRDC Annual Reports - Produce genetically transformed (transgenic) sugarcane plants expressing agriculturally important genes such as resistance to sugarcane mosaic virus and resistance to the herbicide glyphosate.

4.0 INTRODUCTORY TECHNICAL INFORMATION

Improved cane varieties are an attractive solution for many sugar industry problems because they have a low direct effect on the cost of production and are environmentally acceptable. In conventional breeding programs, an increase in the number of criteria for selecting varieties usually will result in slower genetic improvement for each criterion, and slower average progress for all criteria. Genetic engineering offers plant improvement programs the opportunity to meet a broader range of objectives by incorporating novel genes and by efficient manipulation of some plant characteristics.

The aim of this project was to produce cane varieties with resistance to sugarcane mosaic virus (SCMV) and glyphosate herbicides. In the short term, cane varieties which are resistant to glyphosate or other suitable herbicides would increase the profitability of reduced tillage practices which are developing in most canegrowing regions. Similarly, an artificial gene for resistance to SCMV would provide more productive varieties for the Isis district. In the long term, the techniques developed in this project would allow the use of other artificial genes for sugarcane improvement. Genes are being researched in Australia and internationally for control of plant reactions to pests, pathogens and environmental stress, and for plant constituents that affect product quality.

When this project was proposed, negotiations were underway with Monsanto to obtain the gene for resistance to glyphosate. Eventually, Monsanto declined to release this gene to BSES because of the growing market for glyphosate, including its usefulness for `spraying out' sugarcane at the end of the cropping cycle. This did not affect the significance of the project to produce transgenic plants, but did affect one of the outcomes.

5.0 RESEARCH METHODOLOGY

The methodology used in this project is described in detail in Gambley *et al* (1993), Smith and Gambley (1993), Gambley *et al* (in press) and Bryant *et al* (in preparation). A brief description of the materials and methods follows.

Plasmids and DNA preparation
Three plasmids, pEmuGN, pEmuLucN and pESCPN were principally used during the project. Plasmids pEmuGN and pEmu-mcs-N were kind gifts from Dr David Last, CSIRO-DPI, Canberra (Last *et al*, 1991). pEmuGN contains the β-glucuronidase (GUS) gene behind the strong constitutive synthetic monocot promoter `Emu' and a Nos termination signal in bacterial plasmid pUC118. pEmu-mcs-N is a derivative of pEmuGN with the GUS gene replaced by a multiple cloning cassette. Restriction enzyme sites within the cloning cassette were used to construct pESCPN for expression of the coat protein of sugarcane

mosaic virus (Smith *et al*, 1992). Similarly pEmuLucN contains the firefly luciferase gene (Luc) (Smith and Gambley, 1993).

Plasmids were maintained in *E. coli* strains AC001 (recD-) or JC8679 (recBCD-). Plasmids were collected by alkaline lysis and purified by CsCl gradient centrifugation (Maniatis *et al*, 1982). Precipitation of plasmid DNA onto gold or tungsten particles was as described by Gambley *et al* (1993) or Bryant *et al* (in preparation).

Plant material
Axillary buds were aseptically removed from the top metre of mature sugarcane varieties and cultured on MS medium (Murashige and Skoog, 1962) with supplemental kinetin and/or 6-benzylaminopurine (BAP). Callus was initiated from leaf whorl material above the growing point by aseptically removing leaf whorl discs and establishing on MS medium containing 2,4-D. Embryogenic callus was established as described by Taylor *et al* (1992). Meristems and callus were prepared for bombardment as described by Gambley *et al* (1993), Smith and Gambley (1993) and Bryant *et al* (in preparation).

Microprojectile bombardment
The `gun' was essentially as described by Franks and Birch (1991) and used either `Ramset' charges or high pressure nitrogen for macroprojectile propulsion. The following parameters of microprojectile bombardment were investigated for their effect on transformation frequency: microprojectile type; distance the macroprojectile was placed down the barrel; propulsion type; number of bombardments; tissue and sugarcane variety, and plant growth regulator (kinetin/BAP) preconditioning of tissue.

GUS assay
The substrate 5-bromo-4-chloro-3-indoyl-β-D-gluronic acid was used to visualise GUS enzyme activity. An insoluble blue product is produced which indicates GUS activity (Gambley *et al*, 1993).

Luc assay
Leaf segments were placed in scintillation vials containing 0.5 ml of 0.4 mM luciferin in 50 mM Tris-acetate buffer (pH 7.5), and photon production was measured on the ^{3}H channel setting of a Packard 1500 scintillation counter over 16h (Smith and Gambley, 1993).

Western blots
Analysis of protein extracts for expression of the SCMV coat protein was by western blotting whereby the extracted proteins were separated by acrylamide electrophoresis, transferred to a nitrocellulose membrane and hybridised with antisera (Smith *et al*, 1992). Antisera used were anti-SCMVCP (Shukla and Gough, 1984), anti-PVY (potato virus Y) (Boehringer Mannerheim) and anti-PRSV (papaya ringspot virus), a kind gift from Marion Bateson, CMB, QUT. Hybridised bands were visualised with goat anti-rabbit or goat anti-sheep IgG antibodies conjugated with either horse radish peroxidase or alkaline phosphatase. Substrates included luminol, 4-chloro-1-naphthol, BCIP/NBT and Fast Red.

DNA extraction
DNA was extracted from frozen ground tissue with a STE/SDS buffer as described

(Gambley *et al*, 1993; Smith and Gambley, 1993).

PCR primers
The following primers were selected from sequence data and synthesised.

GUS 1000	5'-TTT GCA AGT GGT GAA TCC CGA CCT
GUS 1600	5'-AGT TTA CGC GTT GCT TCC GCC AGT
LUC 721	5'-GTC TCA GTG AGC CCA TAT CCT TGT
LUC 1690	5'-CTG GAG AGC AAC TGC ATA AGG CTA
SCP 000	5'-TCT AGA CCA TGG CTG GAA CAG TCG
SCP 699	5'-CCA TAT CGT GGC ATG TAT CGC TCT

The primer pairs respectively prime the PCR amplification of a 600bp GUS specific DNA fragment, a 970bp Luc specific DNA fragment and a 700bp SCMV coat protein specific fragment when the appropriate template DNA is present in a PCR sample. Full details are in Smith and Gambley (1993).

Southern blotting
PCR products were separated by electrophoresis through a 1% agarose gel, and then capillary blotted onto a nylon membrane using 100 mM NaOH. Blots were baked and then hybridised with the appropriate probe, labelled either with peroxidase for ECL detection (Amersham) or biotin for dioxygenin detection (Boehringer Mannerheim).

6.0 RESULTS

A non-technical summary of the important results follows. Full technical details can be found in Gambley *et al* (1993), Smith and Gambley (1993), Gambley *et al* (in press) and Bryant *et al* (in preparation).

Plasmid quality
The quality of the plasmid DNA was found to be important for high transformation efficiency (Bryant *et al*, in preparation). High quality plasmid DNA prepared by alkaline lysis followed by CsCl-ethidium bromide gradient centrifugation was used for all transformations.

Regeneration
Axillary buds from a number of varieties regenerated shoots although the number of shoots was variety dependant (Gambley *et al*, 1993; Smith and Gambley, 1993). Cytokinin treatment of the meristematic tissue significantly affected the number of shoots both pre- and post-bombardment (Gambley *et al*, in press).

Microprojectile bombardment
Sugarcane meristems can be successfully used as transformation targets and the transgenes detected in the regenerated shoots. The expression of GUS (Gambley *et al*, 1993), Luc (Smith and Gambley, 1993) and SCMV coat protein was detected in regenerated shoots, although the histochemical GUS assay suggested that the regenerated tissue was chimaeric. The distance the macroprojectile was placed down the barrel, the number of bombardments, the variety of sugarcane and the tissue culture plant growth regulator regime were all found to significantly affect the transformation frequency.

Effect of cytokinin regime on transformation frequency
The type and concentration of cytokinin used during tissue culture prior to, and after, microprojectile transformation significantly affected the transformation frequency. The transformation frequency was maximised in plantlets regenerated on MS medium containing 0.5 mg/l kinetin.

Expression of SCMV coat protein by transgenic sugarcane plants
The coat protein of SCMV was expressed in shoots regenerated from microprojectile bombarded meristems. The level of expression was very low and was difficult to visualise on the western blots of protein extracts. The three primary antisera used to detect the presence of the coat protein in western blots had different sensitivities. The anti-SCMVCP was the most sensitive but also cross-reacted considerably with sugarcane proteins, probably the result of sugarcane proteins being present in the purified viral preparation used to raise the antibodies. Anti-PVY and anti-PRSV antisera showed considerably less cross reaction, but were less sensitive at detecting coat protein as these potyviruses have limited coat protein homology with SCMV. The anti-SCMV antiserum was in limited supply so that the sugarcane protein cross reacting antibodies could not be removed, and attempts to source a monoclonal anti-SCMV serum were unsuccessful. A highly purified recombinant SCMV coat protein was purified from *E. coli* containing a fusion protein construct (Bryant *et al*, 1993; Smith *et al*, in preparation). This protein is currently being used to raise rabbit antibodies and this new antiserum should resolve the previous problems.

7.0 DISCUSSION

Transgenic sugarcane plantlets were produced by microprojectile bombardment of sugarcane meristems. Using sugarcane meristems rather than callus as originally proposed, a significant number of the project objectives were fulfilled. This significant modification to the original proposal was discussed with SRDC during the project review in 1992.

Callus and cell suspension cultures of seven cane varieties - Q95, Q137, 66C760, 66C807, 66N2008, 59S55 and Q124 - were established, completing objective 1.

The SCMV coat protein gene construct was successfully incorporated into callus cells regenerated from electroporated callus, meeting objective 2. The recalcitrance of electroporated cells to regenerate into sugarcane plants prevented exploitation of this achievement.

Protocols, initially based on those developed by Dr Birch and colleagues at the University of Queensland, were developed for genetic transformation of sugarcane tissues (mainly meristems) using a microprojectile gun. Protocols, initially based on those developed in BS59S and BS60S as well as BS3S, were developed to prepare cell lines and plant tissue for transformation. Several physical and biological factors influencing microprojectile transformation of both meristems and callus were investigated, and the system was optimised for maximum transformation frequency. This successfully completed objectives 3 and 4.

Protocols where again adapted from BS59S and BS60S, as well as BS3S, for regeneration of transformed plantlets from genetically transformed tissues. Research in this project demonstrated the potential of cytokinin conditioning of tissues prior to, and after, transformation to increase both transformation frequency and the number of transgenic plantlets recovered. Objective 5 of the project was achieved.

Sugarcane plants expressing the coat protein gene of SCMV were produced. The level of expression was very low, probably the result of chimaeric transformants. Plants expressing glyphosate resistance could not be produced as Monsanto declined to release the gene for resistance to BSES. Thus objective 6 was only partially completed, due to reasons beyond our control.

Objective 7 was not fulfilled as transgenic sugarcane plants were not glasshouse or field tested. They were produced late in the third year, and laboratory analysis indicated that the level of gene expression was so low that it would probably not confer field resistance based on published results with other crops. More research will be necessary to elucidate the reason for the low level of expression before the plants can be tested.

8.0 DIFFICULTIES

- Very low level of expression of the coat protein gene in regenerated plants.
- Apparent chimaerism of plants regenerated from bombarded meristems.
- Cross reactivity of antiserum making analysis of transgenics difficult.

9.0 IMPLICATIONS AND RECOMMENDATIONS

Transgenic plantlets expressing the coat protein of SCMV were produced by microprojectile bombardment of sugarcane meristems and subsequent regeneration of transgenic plantlets. This is the first agriculturally significant gene genetically engineered into sugarcane and is a major achievement. However the low level of expression and probable chimaerism of the

transformants preclude exploitation of this achievement. Further research is therefore necessary before the advantages of meristems can be utilised for routine sugarcane genetic engineering. At this time, further research with meristem transformation has been delayed while the callus transformation system developed by Dr Birch and colleagues is used to produce transgenic plants expressing the coat protein of SCMV. This research has been given high priority as production of an SCMV resistant transgenic would be of immediate value to the Australian sugar industry. Somaclonal variation may yet be a problem in transgenic plants regenerated from callus, in which case a meristem transformation system may be the ideal solution.

9.1 Recommendations

- Sequence diversity within the coat protein region of SCMV isolates should be determined and new resistance constructs developed, as very recent results from overseas suggest that coat protein mediated resistance may not work well with a potyvirus like SCMV.
- A better anti-SCMVCP serum is required - this is being addressed and should be available in the near future.
- Continue to develop the meristem transformation system with emphasis on eliminating chimaerism and limiting the potential for somaclonal variation. BSES does not have the necessary resources to research this aspect as well as work on BS94S.
- Every effort should be made to retain the skills and expertise developed and applied in this project within the Australian sugar industry.

10.0 INTELLECTUAL PROPERTY

No patentable developments were made in this project. We reported the first use of sugarcane meristems as a target for microprojectile transformation and that transgenic sugarcane plants can be produced in this way. Transgenic plants produced by microprojectile bombardment and transgenic plants expressing viral coat proteins for synthetic virus resistance are both subjects of patents held by other companies. This issue will need to be addressed before SCMV transgenics can be used in the Australian sugar industry.

11.0 PUBLICATIONS

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11.1 Proposed publications/ presentations

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