BUREAU OF SUGAR EXPERIMENT STATIONS
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FINAL REPORT - SRDC PROJECT BS94S PRODUCTION AND EVALUATION OF SCMV RESISTANT TRANSGENIC SUGARCANE PLANTS DERIVED FROM TRANSFORMED CALLUS

by

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1.0 SUMMARY

We have successfully demonstrated that sugarcane can be genetically engineered for resistance to sugarcane mosaic virus, using the coat protein gene of the virus to mediate resistance. At present the frequency of plants showing moderate to high transgene-mediated resistance to viral infection is low, but the second generation of constructs with stronger promoters should address this issue. Transgenic sugarcane plants of cultivars Q95, Q153 and Q155 that contained a sugarcane mosaic coat protein gene construct under control of the *Emu* promoter were produced. These plants were regenerated from embryogenic callus which had been co-transformed by microprojectile transformation with the coat protein gene construct and the neomycin phosphotransferase II (NPT II) selection gene. The plants were established in the PC2 glasshouse at the Pathology Farm, Eight Mile Plains and were then mechanically inoculated with an inoculum of sugarcane mosaic virus prepared from infected sugarcane grown at the Pathology Farm. One transgenic line of Q155 did not develop mosaic symptoms over the time course of the trial, while another 6 lines of cultivars Q95, Q153 and Q155 showed a delay in the development of symptoms. Molecular analysis of these lines by PCR indicated the presence of both the NPT II and CP genes. Further analysis of selected lines by Southern blot hybridisation indicated the presence of one to three copies of the CP gene.

2.0 BACKGROUND

Sugarcane is a genetically complex plant and combining superior agronomic performance with pest and pathogen resistance into the one cultivar can be very difficult. Further, screening for pathogen resistance generally occurs late in the selection program due to the difficulties in screening large numbers of potential cultivars. Hence, there has already been considerable time and resource invested in selection for elite agronomic phenotypes. Elite clones may need to be discarded at this stage because of susceptibility to one or more pathogens, or the commercial cultivation of these clones may need to be restricted to pathogen-free areas.

Sugarcane mosaic virus (SCMV) is an important pathogen of sugarcane in Australia, and may cause significant yield losses in susceptible varieties. SCMV is a member of the large and economically important viral family, the potyviridae. Pathogen-derived resistance (PDR) genes have been developed to confer resistance to a number of members of the potyviridae. The concept of pathogen-derived resistance was outlined by Sanford and Johnstone (1985). The essence of this concept is if a gene, such as a viral coat protein gene, which is essential to the pathogen but not the host, is expressed out of phase, in the wrong context or in the wrong amount in the host then this may act to disrupt the infection cycle of the pathogen. The concept was first demonstrated by Powell-Abel *et al.* (1986) and although the mechanism may not be as originally proposed, the phenotype of resistance using this approach has been successfully demonstrated in a large number of virus-host combinations. Transgenic plants such as ‘Freedom II’, a squash with transgene-mediated resistance to zucchini yellow mosaic potyvirus are now commercially cultivated in the United States.

Commercial sugarcane cultivars are complex interspecific hybrids between *Saccharum officinarum* and *S. spontaneum*. The cultivars are polyploid in nature, but aneuploidy is common resulting in a very complex genetic background. Backcrossing for the introduction of specific genes without major changes in the genetic background is virtually impossible because of this genetic complexity. Transgenes are an attractive source of resistance in circumstances such as this. The coat protein (CP) coding region of SCMV was developed as a PDR gene in earlier work (BS9S, BS44S) to provide a source of resistance for otherwise viral-susceptible germplasm. Sugarcane, like many members of the Gramineae, had been recalcitrant to genetic transformation. In 1992, the transformation of sugarcane was achieved (Bower and Birch, 1992) using microprojectile transformation of embryogenic callus, and improvements on this technique are now used as the standard transformation system for sugarcane.

The aim of this research was to use this transformation technique to produce SCMV resistant transgenic sugarcane plants expressing the CP gene of the pathogen, and to evaluate the potential of this transgene to provide resistance against SCMV.

3.0 OBJECTIVES

- Produce a range of transgenic plants containing the SCMV coat protein gene using the UQ callus transformation/ regeneration technique.
- Determine co-transformation and co-inheritance frequencies for the introduced genes.
- Determine the level of coat protein produced in the transgenic lines and the relationship between protein level, gene copy number and integration pattern.
- Evaluate the resistance of transgenic plants to SCMV.

4.0 OUTCOMES

- Production of transgenic lines of Q95, Q124, Q137, Q153 and Q155 transformed with the coat protein gene construct.

- Demonstration of transgene-mediated resistance to sugarcane mosaic virus in the first glasshouse trial.

- Preliminary data to indicate that the SCMV resistance transgene should provide resistance against all isolates of SCMV in Australia (based on results in this project combined with data obtained in QUT1S).

- A polyclonal antiserum, prepared by injecting a rabbit with recombinant SCMV coat protein has proved to be a very useful research and diagnostic tool for evaluating viral titre by ELISA in transgenic and other plant samples.

- Molecular analysis indicates there is little correlation between transgene copy number and level of transgene expression (NPT II selection gene).

- Evidence that transgene-mediated resistance in sugarcane is mediated at the RNA level, and not the protein level as originally believed. While understanding the mechanism of resistance is important, it is not essential at this stage as the phenotype of virus resistance is the important outcome.

5.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

5.1 Production of transgenics

Embryogenic calli of cultivars Q95, Q124, Q137, Q153 and Q155 were prepared using established protocols (Bower and Birch, 1992). Briefly, leaf whorls from above the apical meristem were aseptically sectioned transversely and placed onto MS (Murashige and Skoog, 1962) agar which was supplemented with 2,4-D. The callus that was induced was subcultured over a period of two months to produce quantities of white, friable embryogenic callus.

Plasmid constructs, using either the *Emu* (Last *et al.* 1992) or the maize ubiquitin *Ubi* (Christensen and Quail, 1996) promoters were prepared from *E. coli* clones using standard molecular biology protocols (eg Sambrook *et al.* 1989). Maps and descriptions of the plasmids are provided in appendix A. In general plasmids were co-transformed into the callus, usually a 1:1 molar mixture of the plasmid carrying the selectable marker (pEKN or pUKN) and the plasmid carrying the SCMV coat protein transgene (pECP or pUCP). When appropriate, reporter genes (eg pEGN) or other constructs (pUPC) would be used instead of the transgene. Triple plasmid co-transformations were also performed on occasion.

For transformation, the callus was subjected to a pre-bombardment osmoticum treatment of 4 to 20 hours , bombarded with gene constructs delivered by a particle-in-flow gun, and then subjected to a post-bombardment osmoticum treatment of 20 hours. The callus was allowed to 'recover' from the transformation for two weeks prior to selection for transformed embryos on MS media supplemented with the antibiotic geneticin.

After two to three months on the selection regime, shoot development was initiated by transfer of the callus onto MS media without 2,4-D, and placing the cultures in an illuminated tissue culture room.

Plants were adapted to glasshouse conditions using small plastic terrariums, to allow the plants to harden off gradually. Plants were maintained in the PC2 standard glasshouses at the David North Plant Research Centre, BSES, Indooroopilly.

The tissue culture and selection of transformants of Q124 was modified from the standard tissue culture technique. Twice the level of the 2,4-D (6 mg/L) was required for the formation of embryogenic callus. Q124 is also relatively resistant to the antibiotic geneticin and during the post-bombardment selection phase it was necessary to increase the concentration of geneticin in the medium to 60 mg/L). A first batch of regenerated Q124 plants were 'escapes' until this characteristic of Q124 was recognised and

addressed. Based on this result the current practice is to now establish a geneticin 'kill curve' for all new cultivars or varieties introduced into tissue culture for transformation.

5.2 Production of polyclonal antiserum

The full details of this work are provided in Smith *et al*. (1995). Briefly, the coat protein gene was constructed as a fusion with the maltose binding protein (MBP) gene and the fusion protein was expressed in the bacterial host *E. coli.* The fusion protein was purified from the bacterial cell lysate by affinity chromatography, the two domains of the fusion protein separated by specific proteolysis and the coat protein purified from the MBP by ion-exchange chromatography. The highly purified recombinant viral coat protein was injected into a rabbit and the resulting polyclonal serum was found to be specific and of high titre, recognising both native and recombinant SCMV coat proteins with minimal reactions to sugarcane proteins.

An ELISA (enzyme-linked immunosorbent assay) was developed to use this antiserum to screen plant samples for the presence of SCMV coat protein. Briefly, 0.5g of leaf was ground in liquid nitrogen, 5ml of extraction buffer pH9.6 was added and the supernatant collected by centrifugation. The primary antiserum (1µl) was incubated with the sample in 3.5ml of buffer, washed and then the conjugated secondary antiserum (alkaline phosphatase-goat anti-rabbit (4µl/ 4ml) was incubated with the sample. Colour development was with the substrate PNPP (8mg/ 4ml) and the plates were read at 405nm.

5.3 Molecular evaluation of transgenic lines

5.3.1 Initial screening.

Regenerated plants were screened for the presence of the transgene and selectable marker gene by PCR analysis (Table 1). This initial assay was chosen as PCR can detect the presence of transgenes in very small amounts of tissue, permitting sampling for successful transformation and co-transformation while plants were relatively small. Whilst PCR has the advantages of speed and amount of tissue necessary for assay, the method reveals no information about the physical location or number of copies of a particular gene that may be present. Although the number of lines represented in Table 1 is limited, the co-transformation frequency varied between 70 and 100%, indicating that microprojectile co-transformation is an efficient method for introducing unlinked genes (ie genes on two separate plasmids).

Table 1. Initial screening of selected *'Emu'* transgenic lines

Cultivar	Total no.	Constructs		PCR analysis	
			No tested	NPT+	CP+
Q95	29	pECPN/ pEKN	25	24	17
Q153	11	pECPN/ pEKN	11	7	7
Q155	7	pECPN/ pEKN	7	2	2

5.3.2 Genetic analysis

Plants with copies of the transgenes as established by PCR were further analysed by Southern blots (named after the inventor Prof E M Southern). Briefly, intact genomic DNA was extracted from the plants, purified and cut into specific sized fragments using a group of enzymes known as restriction endonucleases. The DNA fragments were separated by size by electrophoresis through an agarose gel, and were then transferred to a nylon membrane so that their relative positions in the gel were retained (the blot). This membrane was then 'probed' with a known piece of DNA (eg transgene) that had been previously labelled with radionucleotides. Under appropriate conditions the probe only recognises pieces of DNA in the genomic DNA that have the same DNA sequence, and 'hybridises' to only these pieces. Because the probe is radioactive, we can detect it's presence by exposing a piece of X-ray film to the membrane. The positions and number of copies of a sequence in the genomic DNA can be deduced from the recorded position of the probe. A Southern blot indicates whether a piece of introduced DNA has become incorporated into the genomic DNA of the plant ('integrated') which is critical for transgene stability and inheritance. In the 'Emu' plants analysed by Southern blotting variable numbers of both transgenes were found (Table 2). Sufficient numbers of Q95 transgenics have been analysed for interpretation. The number of copies of the transgenes introduced varied considerably between the regenerated lines, but the number of copies of the selectable NPTII gene was considerably higher than the number of copies of the CP gene.

Table 2 Genetic analysis of selected *Emu* transgenic lines

Cultivar	**Total no**	**Constructs**		**Southern blot analysis**	
			No tested	Copy no NPT	CP
Q95	29	pECPN/ pEKN	15	1 - 13	0 - 3
Q153	11	pECPN/ pEKN	1	17	3
Q155	7	pECPN/ pEKN	1	1	1

5.3.3 Transgene expression

Expression of genes results in the production of proteins. Proteins can be detected by a variety of methods including assays for proteins with enzymatic activity, radioactive tracers and immunological. In work started in a previous SRDC-funded project (BS44S) and completed in this project, a high titre, highly specific polyclonal antiserum was produced after injection of a rabbit with highly purified recombinant SCMV coat protein (Smith *et al.* 1995). This antiserum is being used in both ELISA and western blot formats to assay for transgene expression. The level of NPTII expression in selected lines was determined by a radioactive ^{33}P enzyme assay (dot/blot). The level of expression of the NPTII varied considerably between lines, essentially spanning a 10 fold level (3 - 24.8 ng/mg) (Table 3). Expression of the CP gene was not detected in any of the lines assayed.

Table 3 Level of expression of the NPTII transgene in selected *'Emu'* transgenic lines

Cultivar	Total no	Constructs	No tested	Concentration of NPTII (ng/mg)
Q95	29	pECPN/ pEKN	29	3.0 - 19.5
Q153	11	pECPN/ pEKN	11	0.0 - 24.8
Q155	1	pECPN/ pEKN	1	23.0

5.4 Glasshouse trial for resistance to SCMV

Fifty-one lines of regenerated transgenic plants of cultivars Q95, Q153 and Q155 were established in a three replicate pot trial in the PC2 Glasshouse at the BSES Pathology Farm. Controls included non-transgenic tissue-cultured Q95, Q153 and Q155. Six weeks after planting the plants were mechanically inoculated with a viral inoculum prepared from the SCMV-infected plants from the Pathology Farm, and then observed for development of symptoms. Six of the lines showed a delay in symptom expression, compared with the controls, indicating increased but not complete resistance to SCMV. Only one line in this experiment showed no mosaic symptoms over the time course of the experiment (40 days after inoculation). All material was destroyed by steam autoclaving at the end of the experiment and the glasshouse prepared for the next set of transgenics to be evaluated (as part of continuing project BS154S). The original clonal material is maintained at BSES, Indooroopilly.

5.5 Concluding discussion

Sugarcane can be genetically engineered with transgenes for resistance to sugarcane mosaic potyvirus. In this project the resistance transgene, the coat protein gene of SCMV, was under the control of the *Emu* promoter. There is now good evidence that this promoter is largely deactivated in differentiated (eg mature plant) sugarcane cells, and this is the most likely reason for the low number of transgenic lines showing good resistance to SCMV infection, although there were a number of lines that showed increased resistance. All current constructs for coat protein mediated resistance are now based on the *Ubi* promoter which appears to be both a stronger promoter and is active in both undifferentiated (eg callus tissue culture) and differentiated (plant) cells. It also became apparent that the tissue culture and selection regimes have to be fine-tuned for the different cultivars. For example, twice the level of 2,4-D is required to induce formation of embryogenic callus in Q124 and this cultivar also shows considerable natural resistance to the antibiotic geneticin resulting in a high number of 'escapes' in early experiments.

Although only a few lines were assessed for expression of the coat protein transgene, no detectable expression was found in any of the samples. This suggests that the transgene-mediated resistance was effected at the RNA, rather than the protein level in the plant cells. We have generated no direct evidence in this project to support this deduction, but the observations and data are similar to reports in the current literature on transgene-mediated resistance to viruses. For this work, elucidation of the mechanism is not nearly

as important as the demonstration of resistant phenotype mediated by the transgene. However, for further work, especially for determining more specifically targeted resistance to sugarcane viruses, it will become important to understand the mechanism of transgene-mediated resistance.

This project had numerous changes of staff during the three years of its operation. These changes significantly affected research progress, and is the main reason why there was only one resistance trial of the transgenic plants. The aim and objectives of the project were essentially completed, but considerably more would have been achieved if there had been more continuity in research staff. During the three years of the project, three Research Officers and two Research Assistants worked to produce mosaic resistant transgenic sugarcane. Only one scientist remained with the project for the three years.

5.6 Recommendations

Pathogen-derived resistance (PDR) genes can be used to genetically engineer virus resistance into sugarcane. Field performance of these plants needs to be assessed: this is part of current SRDC-supported project BS154S. As part of this project we hope to obtain further data to support our initial hypothesis that the transgene sequence used to mediate the resistance will provide field resistance to all isolates of SCMV present in Australia. Further transgenes for resistance to SCMV based on the replicase coding region of the virus have been developed, and transgenic plants containing these constructs will be tested. These replicase constructs should mediate resistance, and having the option of two different constructs for resistance will be important in both negotiations with the coat protein and replicase patent holders and in considerations of deployment of these genes in the field. Discussions with Monsanto on coat protein technology have commenced; discussions with Unilever/ Cornell Foundation should be initiated in the near future.

The successful demonstration of transgene-mediated resistance to SCMV in sugarcane indicates that this technology should be applicable to most, if not all, sugarcane viral pathogens. We have cloned and sequenced most of the genome of Fiji disease virus in SRDC-supported project BS86S so that gene sequences for the development PDR transgenes would be available. Some constructs for FDV transgenic resistance have been designed and developed, and we are encouraged by the SCMV results to begin development of FDV-resistant transgenics, but will require continued support to pursue this aim.

6.0 PUBLICATIONS

Journal papers

G R Smith, R Ford, J D Bryant, R L Gambley, T K McGhie, R M Harding and J L Dale (1995). The expression, purification, and use as an antigen of recombinant sugarcane mosaic virus coat protein. Archives of Virology 140: 1817-1831.

Presentations

Jeff D Bryant, Rebecca Ford, Rhonda L Gambley, Tony K McGhie, Robert M Harding, James L Dale and Grant R Smith (1994). Production of highly specific antiserum to sugarcane mosaic virus following purification of recombinant viral coat protein from *E. coli.* Proceedings of the International Society of Sugar Cane Technologists 4th Sugar Cane Pathology Workshop, p23.

Rhonda Gambley, Jeff Bryant, Niall Masel, Sian Roberts and Grant Smith (1994). Production of transgenic sugarcane plants containing the sugarcane mosaic virus coat protein. Proceedings of the Australian Society for Plant Physiology 34th Conference, No 153.

Rhonda L Gambley, Jeff D Bryant and Grant R Smith (1994) Microprojectile transformation of sugarcane meristems and callus for SCMV resistance. Proceedings of the International Society of Sugar Cane Technologists 4th Sugar Cane Pathology Workshop, p21.

Priya A Joyce, Margaret J Bernard, Kerry A Hickman, Siân E Roberts, Tony K McGhie and Grant R Smith (1995). Production of transgenic sugarcane with genes for resistance to canegrubs and mosaic virus. Proceedings of the Australian Society for Plant Physiology 35th Conference, No36.

Priya A Joyce, Margaret J Bernard and Grant R Smith (1996). Engineering for resistance to SCMV in sugarcane. Proceedings of the 1996 Conferences of the Australian Society for Biochemistry and Molecular Biology and the Australian Society of Plant Physiologists, POS-068-02.

Grant R Smith, Rebecca Ford, Jeff D Bryant, Rhonda L Gambley, Tony K McGhie, Robert M Harding and James L Dale (1995) Expression and purification of recombinant sugarcane mosaic virus coat protein. Proceedings of the 10th Australasian Plant Pathology Society Conference, p36.

7.0 OTHER REFERENCES

R Bower and R G Birch (1992). Transgenic sugarcane plants via microprojectile bombardment. The Plant Journal 2(3):409-416.

A H Christensen and P H Quail (1996). Ubiquitin promoter-based vectors for high level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research 5:213-218.

D I Last, R I S Brettel, D A Chamberlain, A M Chaudhury, P J Larkin, E L Marsh, W J Peacock and E S Dennis (1991). pEmu: an improved promoter for gene expression in cereal cells. Theoretical and Applied Genetics 81:581-588.

T Murashige and F Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

P Powell-Abel, R S Nelson, D Barum, N Hoffman, S G Rogers, R T Fraley, R N Beachy (1986). Delay of disease development in transgenic plants that express the tobacco moasic virus coat protein gene, Science 232:738-743.

J Sambrook, E F Fritsch and T Maniatis (1989). Molecular cloning. A laboratory Manual. 2nd Edition.

J C Sanford and S A Johnstone (1985). The concept of pathogen-derived resistance - deriving genes from the parasites own genome. Journal of Theoretical Biology 113:395-405.

G R Smith, R Ford, M J Frenkel, D D Shukla and J L Dale (1992). Transient expression of the coat protein of sugarcane mosaic virus in sugarcane protoplasts and expression in Escherichia coli. Archives of Virology 125:15-23.

8.0 ACKNOWLEDGMENTS

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9.0 APPENDICES

9.1 Maps of plasmid constructs used

pEmuKN, pEmuCPN, pEmu*uid*A
pUbiKN, pUbiCPN, pUbiPCN

9.2 Copies of publications

G R Smith, R Ford, J D Bryant, R L Gambley, T K McGhie, R M Harding and J L Dale (1995). The expression, purification, and use as an antigen of recombinant sugarcane mosaic virus coat protein. *Archives of Virology 140: 1817-1831.*

Jeff D Bryant, Rebecca Ford, Rhonda L Gambley, Tony K McGhie, Robert M Harding, James L Dale and Grant R Smith (1994). Production of highly specific antiserum to sugarcane mosaic virus following purification of recombinant viral coat protein from E. coli. *Proceedings of the International Society of Sugar Cane Technologists 4th Sugar Cane Pathology Workshop, p23.*

Rhonda Gambley, Jeff Bryant, Niall Masel, Sian Roberts and Grant Smith (1994). Production of transgenic sugarcane plants containing the sugarcane mosaic virus coat protein. *Proceedings of the Australian Society for Plant Physiology 34th Conference, No 153.*

Rhonda L Gambley, Jeff D Bryant and Grant R Smith (1994) Microprojectile transformation of sugarcane meristems and callus for SCMV resistance. *Proceedings of the International Society of Sugar Cane Technologists 4th Sugar Cane Pathology Workshop, p21.*

Priya A Joyce, Margaret J Bernard, Kerry A Hickman, Siân E Roberts, Tony K McGhie and Grant R Smith (1995). Production of transgenic sugarcane with genes for resistance to canegrubs and mosaic virus. *Proceedings of the Australian Society for Plant Physiology 35th Conference, No36.*

Priya A Joyce, Margaret J Bernard and Grant R Smith (1996). Engineering for resistance to SCMV in sugarcane. *Proceedings of the 1996 Conferences of the Australian Society for Biochemistry and Molecular Biology and the Australian Society of Plant Physiologists, POS-068-02.*

Grant R Smith, Rebecca Ford, Jeff D Bryant, Rhonda L Gambley, Tony K McGhie, Robert M Harding and James L Dale (1995) *Expression and purification of recombinant sugarcane mosaic virus coat protein. Proceedings of the 10th Australasian Plant Pathology Society Conference, p36.*