BUREAU OF SUGAR EXPERIMENT STATIONS

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FINAL REPORT – SRDC PROJECT BS86S

CONSTRUCTION OF SYNTHETIC FIJIVIRUS

RESISTANCE GENES FOR USE IN SUGARCANE

by

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SUMMARY

Fiji disease of sugarcane, caused by Fiji disease fijivirus (FDV), is one of the most important diseases affecting sugarcane in Australia. FDV is a member of the reovirus family and has a multipartite genome consisting of ten segments of double stranded (ds) RNA ranging in size from 1.8 to 4.4 kilobasepairs (kbp). The total genome size of FDV is approximately 30 kbp.

Approximately 80% of the FDV genome has now been cloned and sequenced. The majority of the FDV segments characterised to date encode a single protein product, indicated by the presence of a single open reading frame (ORF). Two of the segments, 7 and 9, were found to contain two ORFs each and hence encode two proteins.

The predicted functions of segments 1, 3, 5, 7, 8, 9 and 10 have been assigned based on homology to equivalent segments in related reoviruses, and/or protein expression studies. This data is summarised below:

Segment	Size (bp)	Status (% sequenced)	Predicted function
1	4400	>90%	RNA-dependent RNA polymerase
2	3900	<10%	Unknown
3	3820	100%	Core capsid protein
4	3568	100%	Unknown, non-structural protein
5	3300	>50%	NTP-binding protein
6	3000	>50%	Unknown
7	2194	100%	ORF 1: structural ORF 2: non structural protein
8	1959	100%	NTP-binding/chaperone protein
9	1843	100%	ORF 1: spike protein ORF 2: unknown non-structural protein
10	1819	100%	Outer capsid protein

A construct containing ORF 1 from segment 9 has been prepared and used to transform the sugarcane cultivars, Q117 and Q124. Transgenic plants have been produced for each of these cultivars and are currently being prepared for challenge experiments with FDV.

1.0   BACKGROUND

Fiji disease fijivirus (FDV) is the most important viral pathogen affecting the Australian sugar industry. The last Fiji disease epidemic in the late 1970s and early 80s in the Bundaberg region almost destroyed the southern industry. The overall incidence of Fiji disease in Queensland is currently low, although the disease continues to affect the New South Wales and Rocky Point Mill regions. Fiji disease is also becoming a concern in the Moreton Mill district.

While the disease incidence is low, the potential devastating impact of FDV is reflected in the restrictions still in place within the breeding program on making crosses for which the predicted level of FDV resistance in the progeny is below the defined acceptable level. During the last Fiji epidemic in the Bundaberg region approximately 80% of existing parents were rejected from the breeding program because of inadequate FDV resistance. This program produced a number of FDV-resistant cultivars, although there were some agronomic and sugar yield penalties in these clones. Today it is considered that the loss of overall genetic gain has been recovered in later breeding programs.

Even with a significant emphasis on providing FDV resistance within the breeding programs, there is a number of Q canes today with, at best, average levels of resistance to FDV. For example Q124 has a rating of 6 (on a 1-highly resistant to 9-very susceptible scale), while Q167 an extremely promising cultivar for the NSW region has a rating of 7. It should be noted that NCo310 was originally rated as a 6 for FDV, but the rating was revised to 8 as a result of the Bundaberg epidemic. Furthermore NCo310 is one of the parents of Q124, a cultivar which last year (1997) represented 37.8% of the total tonnes crushed in Australia, and over 80% of the total crush in the central region. It is predicted that the percentage of Q124 in the total crush will continue to rise and will approach 50% of the total Australian crush. Should a Fiji disease epidemic occur in these circumstances, the consequences will be far-reaching and dramatic.

Pathogen-derived resistance (PDR), using genes derived from the pathogen expressed by the plant to mediate resistance, works in sugarcane. In other SRDC-supported research (BS94S and BS154S) it has been demonstrated that resistance can be generated against sugarcane mosaic potyvirus by transforming the plant with the coat protein of the virus (Joyce *et al* (1998) Proc. ASSCT 20:204-210). A similar approach to provide transgenic resistance to FDV has been proposed. PDR transgenes are derived from the genome of the pathogen so information on the organisation and sequence of the pathogen genes is required prior to selection and development of the resistance genes (Smith *et al* 1996). There are many examples of PDR working in various transgenic host-virus combinations, with the most successful strategies utilising either the coat protein or the replicase coding region for construction of the resistance transgene.

The FDV viral particle is more complex than many other plant viruses. It has a double icosahedral outer shell rather than a single coat protein. In addition, both the outer and the inner icosahedral shells contain spikes, named A and B spikes, respectively. The viral nucleic acid is in the form of double stranded RNA (ds RNA) and is located at the centre of the viral particle, within an inner (core) capsid. The icosahedral shells, spikes and the core capsid are potential targets for PDR, in addition to the replicase.

The aim of this project was to characterise the FDV genome by cloning and sequencing the dsRNA, and to identify regions which could then be used in the development of resistance constructs.

2.0   OBJECTIVES

- Produce an FDV library in *E coli*

- Characterise and sequence selected clones

- Identify open reading frames (ORFs) from the FDV sequence data, and use computer programs to predict the size of encoded proteins

- Assign probable gene function to the ORFs by homology and size comparison with known genes for other reoviruses

- Select target sequences for development of synthetic resistance genes

- Express target sequences in *E coli*, tobacco or sugarcane systems as appropriate to confirm gene function.

3.0   OUTCOMES

- An FDV cDNA has been produced in *E coli.*

- Approximately 80% of the FDV genome has been sequenced. Complete sequences are available for segments 3, 4, and 7, 8, 9 and 10.

- Complete ORFs have been identified in segments 3, 4, and 7, 8, 9 and 10 and the size of the encoded proteins have been predicted using computer programs. Partial ORFs have also been identified in segments 1 and 5.

- Potential gene function has been assigned to segments 1, 3, 7, 9 and 10 based on homology to related reoviruses and/or functional analysis.

- The ORFs encoded by segments 7 and 9 have been expressed in *E coli*. Both segments contain two ORFs, and in each segment ORF 1 encodes a structural protein.

- The sequence of segment 9 ORF 1, a putative spike protein, has been cloned into a potential resistance construct containing the Ubi promoter and Nos terminator. This construct has been used to transform Q117 and Q124.

- The antiserum produced against FDV S9 ORF1 is of a high titre and is specific for FDV. This antiserum is potentially useful for FDV diagnosis and a serologically-based diagnostic test for FDV is currently being developed.

- PhD thesis, QUT Burns, P (1998). Molecular characterisation of Fiji disease fijivirus genome segments 5, 7, 8 and 10. Submitted March 1998.
- B AppSc (Hons) thesis, QUT Soo, HM (1997). Molecular characterisation of Fiji disease reovirus genome segment 9. Awarded First Class Honours, June 1997.

4.0 TECHNICAL INFORMATION AND METHODOLOGY

All the methods described in this section are detailed and referenced in the manuscripts in Appendix 8.1.

4.1 FDV extraction method

FDV infection of sugarcane is characterised by the development of galls on the underside of the leaf midrib. Viral dsRNA was isolated from these galls using the following extraction procedure. Briefly, the galls were ground in liquid nitrogen and then stirred for one hour in a NaCl-Tris based buffer with phenol, chloroform, SDS and β-mercaptoethanol. This step releases the viral nucleic acid from the viral particles within the sugarcane tissue. Debris was removed by centrifugation, the dsRNA was bound to cellulose, washed, and then eluted. Finally, the dsRNA was precipitated with isopropanol to provide a concentrated stock of dsRNA for cloning.

4.2 Preparation of an FDV cDNA library

The genome of FDV comprises of ten linear segments of dsRNA, varying in size from the 4.4 kbp (segment 1) to 1.8 kbp (segment10), with a total genome size of about 30 kbp. A complementary DNA (cDNA) library was prepared from the viral dsRNA to permit sequencing of the FDV genome, and provide cDNA clones of the genome for the selection and development of resistance genes.

The dsRNA was denatured at 98°C for eight minutes, then quenched on dry ice. A commercially available cDNA kit (Pharmacia) was used to generate DNA. Briefly, random hexamers were used to prime cDNA, and reverse transcriptase was used to convert the RNA into cDNA. The RNA-cDNA hybrid was then treated with RNase H to degrade the RNA strand. The enzyme DNA polymerase then synthesised the second DNA strand. The dsDNA was then cloned into bacterial plasmid vectors such as pGEM-3ZF, and transformed into the bacterial host *E coli*.

4.3 Characterisation of the FDV library

The FDV cDNA library was initially characterised by assigning clones to individual FDV segments. The 10 segments of viral ds RNA were separated by agarose gel electrophoresis and then transferred to nylon membranes. The cDNA inserts from randomly selected FDV clones were then radiolabelled and used as probes in a standard Northern blotting hybridisation.
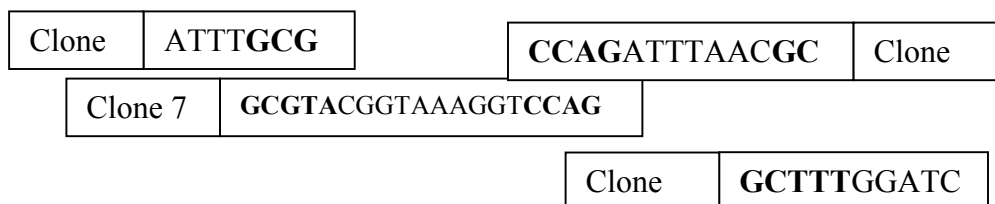
Clones were grouped according to the segment from which they were derived, then selected clones were sequenced. Clones containing overlapping sequences were identified using ANGIS (Australian National Genetic Information Service) programs and contiguous (see Fig 1) sequences were compiled from these clones. In cases where the cloned sequences did not overlap, oligonucleotides were designed from the sequences and reverse transcription-polymerase chain reaction (RT-PCR) amplification was performed. The PCR products were cloned and sequenced, and contiguous sequences were compiled. The FDV terminal sequences were obtained by anchor ligated PCR. Briefly, an oligonucleotide of known sequence, P1, was attached to the end of the FDV segment, then RT-PCR was performed using an oligonucleotide complementary to P1 and an FDV specific oligonucleotide. The PCR products were then cloned and sequenced.

Figure 1   Diagrammatic representation of a contiguous sequence

Contiguous sequence

ATTTGCGTACGGTAAAGGTCCAGATTTAACGCTTTGGATCC

Plasmid clones

Clone	ATTT**GCG**

	CCAGATTTAAC**GC**	Clone

Clone 7	**GCGTA**CGGTAAAGGT**CCAG**

Clone	**GCTTT**GGATC

In molecular biology the term contiguous is applied to a nucleotide sequence that is assembled from the individual sequences of a set of overlapping clones.

4.4   Cloning and expression of FDV ORFs

The FDV ORFs from two segments, 7 and 9, were expressed in *E coli* as fusion proteins with the maltose binding protein using a commercially available kit (pMAL-c2, New England Biolabs). The fusion proteins were purified and injected into rabbits. The rabbits were given three injections at ten day intervals and serum was collected ten days after the final injection. The antisera were then used to probe Western blots of purified viral particles, crude preparations FDV infected tissue and healthy sugarcane (as a control). When the antisera recognised proteins from FDV infected tissue, it was assumed to have been raised against an FDV structural protein.

5.0   RESULTS AND DISCUSSION

5.1 Characterisation of the FDV genome

5.1.1 FDV segment 1 (S1)

Approximately 4.0 kbp of this 4.4 kbp segment has been sequenced. The two ends of this genome segment have not been completed, however the sequence obtained has been translated into an amino acid sequence and analysed. A GDD (G- Glycine, D- aspartic acid) motif has been identified within translated sequence. This GDD motif is a feature of RNA-dependent RNA polymerase enzymes (replicase), the enzyme required for RNA virus replication, therefore S1 is believed to be the FDV replicase.

5.1.2 FDV segment 2 (S2)

S2 was poorly represented in the cDNA library and only one clone containing S2 specific sequence has been identified, yielding very little sequence data. The remainder of S2 will be obtained by Richard McQualter as part of his SRDC-funded PhD project.

5.1.3 FDV segment 3 (S3)

This segment has been completely sequenced and is comprised of 3820 bp. The S3 ORF encodes 1193 amino acids with a predicted molecular weight (Mr) of 137.0 kDa. Sequence analysis of this ORF revealed homology to the inner capsid protein of *Nilaparvata lugens* reovirus (NLRV), hence S3 may encode the core capsid protein.

5.1.4 FDV segment 4 (S4)

This segment has been completely sequenced and is comprised of 3568 bp. The S4 ORF encodes 1146 amino acids with a predicted Mr of 133.3 kDa. Sequence analysis of this ORF revealed homology to NLRV segment 4, a protein of unknown function. The NLRV protein is believed to encode a non-structural protein, hence FDV S4 may also encode a non-structural protein.

5.1.5 FDV segment 5 (S5)

This segment has been partially sequenced from the 5' terminus and a potential ORF has been identified. Sequence analysis revealed a NTP-binding motif suggesting that the ORF may be involved in NTP binding.

5.1.6 FDV segment 6 (S6)

This segment has been partially sequenced, however the clones identified to date do not overlap therefore no characterisation has yet been possible.

5.1.7   FDV segment 7 (S7)

This segment has been completely sequenced and is comprised of 2194 bp. S7 contains two non-overlapping ORFs. S7 ORF 1 encodes 364 amino acids with a predicted Mr of 41.7 kDa, while S7 ORF 2 encodes 307 amino acids with a predicted Mr of   36.7 kDa. The nucleotide and amino acid sequences of S7 exhibited homology to maize rough dwarf fijivirus (MRDV) S6 and rice black streaked dwarf fijivirus (RBSDV) S7, both of which contain two ORFs. The role of ORF 1 for MRDV S6 and RBSDV S7 is unknown. In contrast, FDV S7 ORF 2, MRDV S6 ORF 2 and RBSDV S7 ORF 2 all contain motifs characteristic of a GTP binding protein, suggesting a similar function for the protein encoded by FDV S7 ORF 2.

The S7 ORFs were each expressed independently in *E coli*, and called S7 protein 1 (P1) and S7 protein 2 (P2). Antisera was raised against each protein in rabbits. In Western blot analysis, the antisera to S7 P1 recognised a protein of approximately 40 kDa in FDV infected sugarcane, suggesting that S7 P1 is a structural protein. The antisera to S7 P2 did not recognise any proteins in FDV infected sugarcane, which is consistent with its putative non-structural role as a GTP-binding protein.

5.1.8   FDV segment 8 (S8)

This segment has been completely sequenced and is comprised of 1959 bp. The   S8 ORF encodes a protein of 594 amino acids corresponding to a Mr of 69 kDa. FDV S8 contained both an NTP-binding motif and a chaperone protein motif suggesting that, like the protein encoded by S5, the S8 protein may be involved in NTP-binding.

5.1.9   FDV segment 9 (S9)

This segment has been completely sequenced and is comprised of 1843 bp. S9 contains two non-overlapping ORFs, S9 ORF 1 and S9 ORF 2. S9 ORF 1 encodes 335 amino acids, corresponding to a Mr of 38.6 kDa, while S9 ORF 2 encodes 208 amino acids, corresponding to a Mr of 23.8 kDa.

The S9 ORFs were each expressed independently in *E coli* and called S9 P1 and   S9 P2. Antisera was raised to each protein. The antisera to S9 ORF 1 recognised a protein of approximately 39 kDa in FDV infected sugarcane, suggesting that S9 P1 is a structural protein. The antisera to S9 P2 did not recognise any proteins in FDV infected sugarcane, suggesting a non-structural role (Soo 1997, Soo *et al* 1998). The size of S9 P1 is consistent with that of a spike protein from another reovirus, rice ragged stunt reovirus, although no homology was observed between the two proteins.

5.1.10   FDV segment 10 (S10)

This segment has been completely sequenced and is comprised of 1819 bp. The S10 ORF encodes 554 amino acids corresponding to a Mr of 63 kDa. FDV S10 exhibited

significant sequence homology to MRDV S10, RBSDV S10 and NLRV S8. Characterisation of NLRV S8 has indicated that it encodes an outer capsid protein, hence FDV S10 may fulfil a similar role.

5.2 Candidate FDV sequences for use in PDR

The characterisation of the FDV genome has led to the selection of several FDV ORFs as potential transgenes to develop PDR constructs. The sequences of S3, S7 ORF 1, S9 ORF 1 and S10 all encode probable structural proteins, as determined by sequence comparisons or protein expression experiments (Sithisarn-Burns *et al* 1996, Burns 1998). In the potyvirus family, structural proteins, in the form of the coat protein, have been successfully used in PDR strategies.

An alternative PDR strategy has been to use the viral replicase sequence as a transgene. The FDV replicase is believed to be encoded by S1. Although the S1 sequence is currently incomplete, recent research suggests that a sequence of approximately 1 kbp is sufficient to trigger PDR. Therefore, resistance constructs based on the FDV replicase can now be prepared from the existing S1 sequence.

5.3 Development of FDV transgenic plants

The sequence of S9 ORF 1, a potential spike protein, has been cloned into a plant transformation vector. Expression of this ORF is under the control of the maize polyubiquitin (Ubi) promoter and the Nos terminator from *Agrobacterium*.

This construct was transformed into cultivar Q117. Several lines have been regenerated and the transgenic plants have been ratooned and are ready to be challenged with FDV. The S9 construct has also recently been used to transform the FDV susceptible Q124 cultivar and transgenic plants are now being produced by Richard McQualter as part of his SRDC-funded PhD project.

6.0 IMPLICATIONS AND RECOMMENDATIONS

Recent success by BSES to develop SCMV-resistant and canegrub-tolerant variants of agronomically elite sugarcane cultivars demonstrates that genetic engineering for pest and pathogen resistance is a viable method to recover valuable germplasm that lacks specific resistance. Fiji disease is a significant threat to the Australian sugar industry, especially as the percentage of Q124 continues to grow for the next five years. Fiji disease is currently threatening the future of Q124 in Rocky Point, hence FDV-resistant Q124 would be of immense value to the industry.

The aims of this project have been met successfully and several potential transgene sequences have been identified. Some of the FDV sequence data generated from this project is currently being used to develop FDV-resistant sugarcane in the SRDC-funded

PhD project of Richard McQualter. Due to the complexity of the FDV virions, genome and life cycle, the most appropriate transgene to confer resistance is unknown. Therefore, a variety of different transgenes will need to be used in order to maximise the chances of success. More scientists and resources will therefore be required if FDV-resistant sugarcane plants are to be realistically delivered to the industry within 5-10 years.

7.0 ACKNOWLEDGMENTS

The practical work involved in this project was carried out at QUT by Parichart Burns, Dr Jennifer Handley, Hui Meng Soo and Mario Maugeri. We also wish to thank Barry Pearce and Gareth Disley of the BSES Pathology Farm, Eight Mile Plains for their help in maintaining FDV-infected cane for the project.

8.0 APPENDICES

8.1 Publications

8.1.1 Soo, HM, Handley, JA, Maugeri, MM, Burns, P, Smith, GR, Dale, JL & Harding, RM (1998). Molecular characterisation of Fiji disease fijivirus genome segment 9. *Journal of General Virology* (accepted subject to minor revision).

8.1.2 Smith, GR, Joyce, PA, Handley, JA, Sithisarn, P, Maugeri, MM, Bernhard, MJ, Berding, N, Dale, JL & Harding, RM (1996). Genetically engineering resistance to sugarcane mosaic and Fiji disease viruses. In *Sugarcane*: *Research Towards Efficient and Sustainable Production* pp138-140.

8.1.3 Burns, P (1998). Molecular characterisation of Fiji disease fijivirus genome segments 5, 7, 8 and 10. PhD thesis, QUT. *(not included)*

8.1.4 Soo, HM (1997). Molecular characterisation of Fiji disease reovirus genome segment 9. BAppSc(Hons) thesis, QUT. *(not included)*

8.2 Presentations

8.2.1 Sithisarn-Burns P, Maugeri, MM, Dale, JL, Smith, GR & Harding RM (1996). Cloning and preliminary sequence analysis of the sugarcane Fiji disease virus genome. Proceedings of the 10th International Congress of Virology, Jerusalem. p184.

8.3 FDV sequences

FDV segment 3
FDV segment 4

FDV segment 5
FDV segment 7
FDV segment 8
FDV segment 10