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**FINAL REPORT - SRDC PROJECT BSCS1
AN ASSESSMENT OF THE APPLICATION OF
DNA MARKERS TO STUDIES OF
GENETIC DIVERSITY AND
MARKER ASSISTED SELECTION IN SUGARCANE**

by

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SUMMARY

Sugarcane is a complex aneuploid, polyploid, interspecific hybrid. At the time this project began, molecular marker research was in its infancy in sugarcane. The first objective of the project was, therefore, to adapt molecular marker technologies to this complex plant. This has been successfully achieved. Beginning with RAPDs and RFLPs, new technologies, such as AFLPs, STS, DAFs and SSRs, have been successfully adapted for use in sugarcane and have been successfully used to achieve the following results. (*See Appendix 1*)

BSES has a substantial germplasm collection of breeding canes, *Saccharum* spp. and *Erianthus* spp. This collection is important in both the breeding of commercial cane varieties and in wide crosses to broaden the genetic base of cultivated sugarcane. Diversity studies were initiated in this material in an attempt to partially characterise the germplasm and to provide information to the breeders on the level of diversity in the collection. This information, in turn, can be used to identify potential parents for future crossing studies through maximising elite progeny production and minimising the effects of inbreeding.

Genetic diversity studies were carried out on the Meringa breeding cane collection using RAFs (Radio-labelled Amplified Fragments) and the highly polymorphic sorghum sb1-10 markers. In these experiments, the parents of 20 very good crosses and 20 very bad crosses were chosen for molecular analysis involving 200 randomly sampled RAF markers and the sb1-10 markers. The extent of genetic diversity between parents of good and bad crosses was then compared with the net merit grade (overall agronomic performance) of the progeny. No positive or negative correlation was observed, and the net merit grade of progeny from crosses can not be predicted from the extent of genetic diversity between the parents. The same conclusion was drawn from both marker technologies. Amongst the 63 clones, the level of similarity between any two clones ranged from about 70 to 85% as assessed by RAFs. However, RAFs may underestimate the level of genetic diversity in the collection as the level of similarity between any two of the 63 clones ranged from about 50 to 100% as assessed by the sb1-10 marker. Genetic diversity studies were also carried out on a subset of the CSR breeding cane collection using RAPDs (Randomly Amplified Polymorphic DNA). Similarly, no correlation was found between genetic diversity and family performance. However, a weak correlation ($r = 0.3$) was found between genetic diversity and specific combining ability, a component of performance related to the specific interaction between two parents. Further work in this area is underway.

Two hundred accessions of *S. officinarum* have been imported into Australia to broaden the genetic base of sugarcane. Unfortunately, many developed disease symptoms before release from quarantine and the entire collection had to be destroyed. Leaf material was collected from these clones and is being stored at the BSES laboratory at Indooroopilly. Using the highly polymorphic sorghum sb1-10 marker system, 50 *S. officinarum* clones in the Meringa collection were assessed, and a high level of diversity was observed.

The genus *Erianthus* is closely related to *Saccharum* and is thought to have been involved in its evolution. Wide crosses between *E. arundinaceus* and sugarcane are being attempted both to broaden the genetic base and to introgress favourable agronomic traits, such as drought and flooding tolerance, vigour, *Pachymetra* resistance and others, into sugarcane. Sixty-five accessions of *Erianthus*, from 11 species, were evaluated using RFLPs (Restriction Fragment

Length Polymorphisms) with rDNA and random probes. These studies found that levels of genetic diversity varied markedly within and between species and that some agronomically important species had very low levels of genetic diversity. The study also provided species-specific markers that could differentiate between morphologically indistinguishable species and allowed unclassified and mis-classified accessions to be classified.

Five sugarcane crosses have been established. Following preliminary evaluation for agronomic traits, two crosses were identified with sufficient progeny as potentially useful for developing marker-assisted breeding in sugarcane. The progeny of one of the crosses, Q117 x 74C42, have been screened with RAPD markers to verify their authenticity.

Numerous *Saccharum* x *Erianthus* crosses had been made by BSES in an attempt to introgress agronomically useful traits from *Erianthus* into sugarcane and morphological analysis of the progeny suggested that many of the progeny were hybrids. Molecular analyses were performed on these progeny to demonstrate that only one cross, a cross between *S. officinarum* x *E. arundinaceus*, had given hybrid progeny. Initially RFLPs and RAPDs were used to screen the crosses. However, given the laboriousness of the former and the difficulties in scoring of the latter, several new techniques have been developed to more quickly and reliably identify hybrid progeny and allow *Erianthus* chromosomes to be followed in future generations of introgression programs. The first technique to be developed used primers from the 5S DNA locus to amplify fragments of different size in *Erianthus* and *Saccharum* species, permitting rapid identification of hybrid and selfed progeny. The 5S PCR test has been widely used in *Erianthus* introgression programs around the world. However, we adapted this test for use on intact leaf tissue, thereby enabling hundreds of progeny to be screened at the breeding station. Rapid techniques for obtaining DNA from both fresh and dried leaf material have also been developed. Several *Erianthus*-specific repeated sequences have been obtained and primers designed to these sequences have also been used to confirm hybrids. More recently, a total genomic method of identifying and quantifying *Erianthus* introgression has been developed.

A PCR test on intact leaf tissue was also developed for the detection of *S. officinarum*/ *S. spontaneum* hybrids. This test uses the sorghum SSR marker (sb1-10) that detects highly polymorphic loci in sugarcane. This PCR test on intact tissue is also extremely useful for the genetic fingerprinting of sugarcane varieties, and for assessing genetic diversity in sugarcane germplasm collections.

Molecular markers have also been used to assess the genetic integrity of sugarcane seedlings regenerated from tissue culture. Interestingly, we demonstrated that DNA fragments can be lost from sugarcane during the tissue culture and transformation process, but more importantly, that the loss of RAF fragments was strongly correlated with loss of agronomic performance in transgenic lines.

1.0 BACKGROUND

Modern sugarcane clones grown for commercial production are complex interspecific hybrids. They resulted from the nobilisation of the wild species *S. spontaneum* by repeated backcrossing to the noble species *S. officinarum*. Relatively few plants are thought to have been involved. Consequently, the genetic base of sugarcane is very narrow. In addition, the cytology of the modern hybrids is complex. The contributing species are themselves polyploid or of a polyploid series. Modern hybrids have elevated chromosome numbers in the range $2n = 100-125$. Some 5-10% of these are of *S. spontaneum* origin. Their inclusion in selected interspecific hybrid progeny is the result of what is thought to be a random process and currently is beyond the control of plant breeders. However, while *S. spontaneum* can contribute highly desirable traits to nobilised progeny, this is not without cost. The transfer of whole chromosomes means undesirable traits are also frequently transmitted. Valuable genes may also be introgressed into sugarcane varieties from wild relatives such as *Erianthus*.

Molecular markers are widely used in crop production in a variety of applications, including fingerprinting, genetic diversity analyses, introgression, marker-assisted selection and in map-based gene cloning. Molecular markers have considerable potential to increase the efficiency of sugarcane breeding. Sugarcane breeding is expensive and time consuming – a commercial variety takes 10-15 years to develop at a cost of approximately \$1m. The use of well developed molecular markers technology will reduce the average time and cost of producing varieties. This project aims to develop molecular markers technology for sugarcane for use in several applications. For example, they can be used to measure the genetic diversity among sugarcane and related species, allowing better management of these genetic resources. Information about genetic diversity can be used to:- reduce the number of canes retained in the collection while minimising the loss of useful genes; confirm the identity and ancestry of parental varieties; identify duplications in germplasm collections; screen new accessions before they enter the germplasm collection; develop a better understanding of sugarcane genetics and make informed selection of parents for use in crossing, enabling the genetic base of modern sugarcane varieties to be broadened.

Molecular markers can also be used to assess the extent of genetic change during tissue culture. Tissue culture is a useful method for rapidly propagating a desirable genotype and is a critical step in the transformation process. Genomic changes through tissue culture has been shown to be a problem in some cases. It is essential that the extent of genomic change during tissue culture be assessed if genetic transformation and tissue culture are to be widely used in sugarcane production.

Marker-assisted selection, wherein molecular markers act as reporters for economically important traits, will also speed up screening for these traits and allow rapid genetic progress. Markers will enable desirable varieties to be identified early in the breeding program. This technology will allow specific segments of DNA, the genetic material of heredity, to be used as genetic markers. These DNA markers will be easy to detect and can be correlated with trait expression, so selection for or against particular markers will result in a response in that particular trait. The DNA of a plant remains constant throughout the life cycle of the plant. Thus, large numbers of plants can be easily screened at any time for DNA markers correlated with traits that are only expressed under particular environmental conditions (eg disease,

frost) or at particular developmental stages of the plant life cycle (eg flowering, maturity). At present, screening lines for resistance to diseases such as Fiji disease and Pachymetra root rot is so expensive that only commercial varieties are assessed, prior to release.

The successful development and application of DNA markers in sugarcane breeding could revolutionise the genetic understanding and rate of improvement of sugarcane productivity.

2.0 OBJECTIVES

1. Adapt and assess various molecular marker technologies including RFLPs, RAPDs, AFLPs and SSR-PCRs for use in sugarcane.

Over Achieved: RFLPs, RAPDs, AFLPs and SSR-PCR technologies have been successfully adapted for use in sugarcane and successfully applied to investigate genetic diversity, hybrid authenticity, and tissue culture integrity. In addition, new technologies have been developed, such as RAFs , STS-PCR and genomic slot-blots.

2. Assess levels of diversity in the Meringa breeding cane collection.

Over Achieved: Breeding canes corresponding to 20 good crosses and 20 bad crosses have been screened using RAFs and levels of genetic diversity measured. Preliminary attempts to correlate genetic diversity with performance have also been attempted, but no obvious correlation has been found. In addition, levels of diversity in a subset of the CSR breeding collection have been assessed and no correlation between diversity and performance was identified. A weak correlation between diversity and specific combining ability has been identified and is being pursued.

3. Assess levels of diversity within 200 *S. officinarum* accessions, recently introduced into Australia.

Partly Achieved: Unfortunately, before these clones were released from quarantine, many of them developed disease symptoms and the entire collection had to be destroyed. Fifty *S. officinarum* clones in the Meringa collection were, however, assessed with the sorghum sb1-10 SSR marker.

4. Assess levels of diversity within *Erianthus* and between *Erianthus* and *Saccharum* species, utilising accessions from the Meringa germplasm collection.

Over Achieved: RFLPs using rDNA and random probes were used to assess levels of genetic diversity in the Meringa germplasm collection and to measure diversity within and between species. In addition, New World *Erianthus* accessions from the USDA collection in Houma, Louisiana, were assessed.

5. Confirm and analyse new *Erianthus* introgression populations.

Over Achieved: Old and new *Erianthus* introgression populations were analysed. Initially, RFLPs were utilised. More rapid techniques using STS-PCR (5S DNA and *Erianthus*-specific repeated sequences) and genomic dot blots have been developed for rapid screens and screens of large numbers of progeny. Techniques involving whole plant tissue have also been developed.

6. Conduct crosses to establish populations for use in subsequent development of marker-assisted selection in sugarcane and verify authenticity of crosses by molecular marker analysis of progeny.

Achieved: Five crosses were initially established. Following preliminary evaluation of the crosses, two crosses were selected for future activities. One of these crosses has been evaluated for authenticity using RAPD markers.

7. Assess the genetic integrity of sugarcane seedlings regenerated from tissue culture using molecular markers.

Over Achieved: RAFs were used to investigate genetic integrity in transgenic plants derived from two sugarcane varieties, Q117 and Q155. Three transgenics from each of these varieties were compared to the parent variety for genetic integrity and agronomic performance. This work was done in collaboration with Dr Robert Birch (Botany, UQ) who supplied the transgenic material, and Dr Mike Cox (BSES) who assessed the transgenic lines in the field. We deliberately chose transgenic lines that showed a range of agronomic performance in the field. Interestingly, we demonstrated that RAF fragments can be lost from sugarcane during the tissue culture and transformation process, but more importantly, that the loss of RAF fragments was strongly correlated with loss of agronomic performance in transgenic lines.

3.0 METHODOLOGY

1. Marker techniques

Protocols for marker techniques developed as part of BSCS1 are contained in Appendix 1. These techniques include DNA isolation from intact tissue, RAPD analysis, RFLP analysis, RAF analysis, AFLP analysis, genomic slot blot analysis etc.

2. Levels of diversity

a) Within the Meringa germplasm collection.

To assess the levels of genetic diversity in the Meringa germplasm collection, DNA was extracted (as described by Carroll *et al.*, 1995, Genetics 139, 407-420) from parents corresponding to 20 very good or 20 very bad crosses. Some parents were represented in more than one cross, and therefore the total number of parent clones included in the analysis was 63. RAFs with four separate oligonucleotide primers yielded 200 markers on the 63 clones. Individual clones were scored for either presence or absence of the

fragment. The genetic diversity between individual pairs of parents was compared with the net merit grade of the progeny from the cross.

b) Within the CSR germplasm collection.

Thirty-two parental lines were selected from the CSR breeding program. These lines had been involved in 38 crosses for which performance data were available. The 32 lines were screened with approximately 100 polymorphic RAPD markers and pairwise similarity coefficients were calculated using the method of Dice, 1945, Ecology, 26:297-302. A simple correlation between similarity between parents of crosses and performance of that cross was calculated. A correlation between similarity between parents of a cross and the deviation between the cross yield and the mean yield of all crosses involving that parent was then calculated.

3. Levels of diversity within *S. officinarum*

As described earlier, before these clones were released from quarantine, many of them developed disease symptoms and the entire collection of 200 clones had to be destroyed. Leaf tissue from these clones has been frozen for later analysis if required. Fifty *S. officinarum* clones in the Meringa collection were, however, assessed with the sorghum sb1-10 SSR marker. This involved PCR analysis on alkali treated intact leaf tissue.

4. Levels of diversity within *Erianthus*

- a) Ribosomal DNA variation – Sixty-two *Erianthus* accessions, representing 11 species, and 15 *Saccharum* accessions from two species were assessed for variation at the 18S-26S and 5S rDNA loci, as described in Besse *et al.*, 1996, Theor. Appl. Genet. 92:733-743. Genomic DNA was digested with restriction enzymes, *Eco*RI, *Bam*HI, *Sac*I, *Bst*EII, *Eco*RV, *Xba*I and *Bgl*II, blotted onto nylon membrane and hybridised with radio-labelled 18S-26S and 5S rDNA probes. Hybridisation patterns amongst the accessions were scored and compared.
- b) RFLP variation – Sixty-five *Erianthus* accessions, representing seven species and 14 *Saccharum* accessions from two species were studied by RFLP analysis, as described in Besse *et al.*, 1997, Euphytica, 93: 283-292. Genomic DNA was digested with restriction enzymes *Eco*RI, *Dra*I and *Hind*III, blotted onto nylon membrane and hybridised with 14 maize RFLP probes. The resulting 112 RFLP fragments were scored for presence or absence in each accession. Pairwise genetic distances were estimated using the method of Dice, 1945, Ecology, 26:297-302. Cluster analysis was performed using the McQuitty similarity analysis of SAS. A Principal Component Analysis was also performed using the ProcMDS analysis from SAS.

5. Analysis of *Erianthus* introgression populations

- a) *Erianthus* populations – Crosses have been made between sugarcane and *Erianthus* species and between *S. officinarum* and *E. arundinaceus* at Meringa. This material has been screened with a range of marker types to identify hybrid material.
- b) 5S rDNA PCR test and DNA isolation from intact tissue. Primers specific to the 5S rDNA were used to amplify 5S DNA repeat units from *Saccharum* species and *Erianthus* species, as described by D'Hont *et al.*, 1995, Theor. Appl. Genet. 91:320-326. Template for PCR was prepared using a modification of the Klimyuk procedure, as described in Carroll *et al.*, 1995, Genetics 139:407-420.
- c) RAPD tests. RAPD primers were identified which produced *Erianthus*-specific bands. These primers were then used to screen putative hybrids.
- d) *Erianthus* repeated sequences. *Erianthus*-specific repeated sequences were isolated as described in Besse and McIntyre, 1998, Genome, in press. These sequences were used in genomic slot blot tests. In addition, primers were designed to the sequences and used to amplify *Erianthus*-specific bands, as described.
- e) Genomic Slot Blots. Genomic slot blots were used to identify *Erianthus x Saccharum* hybrids, as described in Besse *et al.*, 1997, Genome, 40:428-432.

6. Production of future mapping populations and verification of authenticity

Five crosses were made using standard techniques. The parents of the crosses were selected as being likely to differ in rust reaction and segregate for other traits such as CCS and yield.

7. Genetic change in tissue culture

RAFs were implemented to assess the genetic integrity of transgenic sugarcane derived from two varieties, Q117 and Q155.

4.0 RESULTS AND DISCUSSION

1. Marker techniques

Protocols for marker techniques developed as part of BSCS1 are contained in Appendix 1. These techniques include DNA isolation from intact tissue, RAPD analysis, RFLP analysis, RAF analysis, AFLP analysis, and genomic slot blot analysis.

2. Levels of diversity within:

A. The Meringa germplasm collection.

To assess the levels of genetic diversity in the Meringa germplasm collection, 63 clones corresponding to the parents of 20 good and 20 bad crosses were assessed with 200 RAF markers. Twenty five percent of the markers were not polymorphic between the 63 clones. For the remaining polymorphic markers (75% of the total), the percent representation for each marker in the 63 clones ranged from 2% for one marker to 98% for another marker. On average, polymorphic markers were represented in $56 \pm 28\%$ of the clones. Using RAFs, the highest degree of similarity between any 2 clones was 85%, and the lowest degree of similarity between any 2 clones was 70%. However, RAFs may underestimate the level of genetic diversity in the collection as the level of similarity between any two of the 63 clones ranged from about 50 to 100% as assessed by the sb1-10 marker.

B. The CSR germplasm collection.

Thirty-two parental lines were selected from the CSR breeding program. These lines had been involved in 38 crosses for which performance data, CCS and TCH, were available at two sites. The 32 lines were screened with approximately 25 RAPD primers, generating approximately 100 polymorphic RAPD markers. Polymorphic bands, only, were scored for their presence or absence in the 32 lines. Pairwise similarity coefficients were calculated using the method of Dice, 1945, Ecology, 26: 297-302. These similarity coefficients are relative measures of similarity only, as monomorphic bands were not included in the analysis and, thus, levels of similarity are underestimated. The relative similarity coefficients ranged from 0.482 – 0.747. A simple correlation between similarity between parents of crosses and performance of that cross was calculated. The magnitude of the correlation varied with sites but was not significant at either site or when averaged across sites.

Table 1. Correlation between similarity and performance

Site	CCS	TCH
Site 1	0.010	0.014
Site 2	-0.083	-0.329
Across sites	-0.036	-0.152

A correlation between similarity between parents of a cross and the deviation between the cross yield and the mean yield of all crosses involving that parent was then calculated for TCH. The correlation was significant at Site 2 and across sites, but not at site 1.

Table 2. Correlation between genetic similarity and the difference between specific cross cane yield and parental cross average cane yield measured

Site	TCH
Site 1	-0.12
Site 2	-0.38 **P<0.001
Across sites	-0.30 *P<0.05

C. *S. officinarum*

Fifty *S. officinarum* clones in the Meringa collection were, however, assessed with the sorghum sb1-10 SSR marker. This demonstrated that there was a high degree of polymorphism at these loci between the *S. officinarum* clones.

D. *Erianthus*

- a) Ribosomal DNA variation – Sixty-two *Erianthus* accessions, representing 11 species, and 15 *Saccharum* accessions from two species were assessed for variation at the 18S-26S and 5S rDNA loci, as described in Besse *et al.*, 1996, *Theor. Appl. Genet.* 92:733-743. Genomic DNA was digested with restriction enzymes, *EcoRI*, *BamHI*, *SacI*, *BstEII*, *EcoRV*, *XbaI* and *BglII*, blotted onto nylon membrane and hybridised with radio-labelled 18S-26S and 5S rDNA probes. Hybridisation patterns amongst the accessions were scored and compared. RDNA units in *Erianthus* sect. *Ripidium* exhibited an additional *BamHI* site compared to *Saccharum*. Six different rDNA units were identified in *Erianthus* sect. *Ripidium*, differing by restriction site positions and/or length. The Indonesian and Indian forms of *E. arundinaceus* gave different restriction patterns. The Indonesian forms of *E. arundinaceus* gave patterns which were similar to that of *E. bengalense*, from India, while the Indian forms of *E. arundinaceus* gave patterns which were similar to the patterns of *E. procerus*, also from India. The two 2n=20 species, *E. elephantinus* and *E. ravennae* could also be differentiated at this locus. Two of the New World *Erianthus* species studied, *E. rufipilus* and *E. longisetosus*, appeared more like *Erianthus* sect. *Ripidium*, supporting their proposed transferral. However, New World species *E. trinii* and *E. brevibardis* appeared more like *Misanthus sinensis* and *S. spontaneum*, respectively, which is in agreement with chromosome number and morphological information for these species (D. Burner, pers. comm.). The 5S rDNA units showed length and restriction site differences between *Erianthus* and *Saccharum*. No differences were observed within the *Erianthus* genus.
- b) RFLP variation – Sixty-five *Erianthus* accessions, representing seven species and 14 *Saccharum* accessions from two species were studied by RFLP analysis, as described in Besse *et al.*, 1997, *Euphytica*, 93:283-292. Genomic DNA was digested with restriction enzymes *EcoRI*, *DraI* and *HindIII*, blotted onto nylon membrane and hybridised with 14 maize RFLP probes. The resulting 112 RFLP fragments were scored for presence or absence in each accession. Pairwise genetic distances were estimated using the method of Dice, 1945, *Ecology*, 26:297-302 and the McQuitty procedure of SAS was used to cluster the accessions. A Principal Component Analysis was also performed using the ProcMDS analysis from SAS. Using the method of Dice, an intergeneric distance (1-F) of 0.748 was calculated between *Erianthus* and *Saccharum*. Within the *Erianthus* collection, the greatest distances were found between *E. elephantinus* or *E. ravennae* (the two 2n=20 species) and the rest of the *Erianthus* collection. The smallest distances were found amongst the *E. arundinaceus* collected in India (1-F=0.005). The cluster analyses separated *E. arundinaceus* into two groups, consistent with their geographical origin and chromosome number. The first group contained individuals from Indonesia with 2n=60 chromosomes. The second group contained accessions from India with 2n=30,40 chromosomes and clustered with other 2n=40 chromosome accessions from India, namely *E. bengalense* and *E. procerus*.

3. Analysis of *Erianthus* introgression populations

- a) *Erianthus* populations – Crosses have been made between sugarcane and *Erianthus* species and between *S. officinarum* and *E. arundinaceus* at Meringa. This material has been screened with a range of marker types to identify hybrid material. New techniques have also been developed to enable *Erianthus* hybrids to be rapidly identified and to allow *Erianthus* material to be followed in subsequent generations of the introgression program.
- b) 5S rDNA PCR test and DNA isolation from intact tissue. Primers specific to the 5S rDNA were used to amplify 5S DNA repeat units from *Saccharum* species and *Erianthus* species, as described by D'Hont *et al.*, 1995, Theor. Appl. Genet. 91:320-326. Template for PCR was prepared using a modification of the Klimyuk procedure, as described in Carroll *et al.*, 1995, Genetics 139:407-420. This DNA isolation procedure is extremely useful as it allows DNA of suitable quantity and quality for PCR analyses to be very rapidly isolated, enabling large numbers of individuals to be rapidly screened. In addition, an alternative DNA isolation procedure has been devised for rapid isolation of DNA for PCR from lyophilised tissue. This method is described in Besse and McIntyre, 1996, Sugar 2000 Symposium, Sugarcane: research towards efficient and sustainable production, pp59-61. These DNA isolation techniques, together with the 5S rDNA PCR protocol, reliably allow large numbers of progeny to be screened and *Saccharum x Erianthus* hybrids to be detected.
- c) RAPD tests. The 5S DNA locus is located on 1 pair of chromosomes only. It was, therefore, deemed useful to identify other *Erianthus*-specific markers to tag other *Erianthus* chromosomes and confirm the true hybrid nature of putative *Saccharum x Erianthus* hybrid progeny. Numerous RAPD primers were screened. Several primers were identified which produced reliable *Erianthus*-specific bands. Several of these primers were then used to screen putative hybrids from a cross between *S. officinarum x E. arundinaceus*, produced by Copersucar in Brazil. The useful primers included Operon primers: AN1, AN5, AN6, AN7, AN10, AN14, AN15, AN16, AN18, and C12.
- d) *Erianthus* repeated sequences. *Erianthus*-specific repeated sequences were isolated as described in Besse and McIntyre, 1998, Genome, in press. These sequences were also used in genomic slot blot tests, as described in Besse *et al.*, 1997, Genome, 40:428-432. These repeated sequences showed varying degrees of repetitiveness and different patterns of distribution. Two of the sequences were low to medium copy repeated sequences and appeared to be present at discrete locations in the *Erianthus* genome. Another sequence was also a low to medium copy sequence but appeared to be more dispersed in location, with some tandem arrays identified. The fourth sequence was highly repeated and dispersed. *In situ* hybridisation confirmed the dispersion of this sequence along the length of, but not at the telomeres of, most chromosomes of two *Erianthus* species. In addition, primers were designed to the sequences. The primers designed to two of the repeated sequences successfully amplified *Erianthus*-specific bands.
- e) Genomic Slot Blots. Genomic slot blots were developed to rapidly identify *Erianthus x Saccharum* hybrids, as described in Besse *et al.*, 1997, Genome, 40:428-432. This technique is based on the genomic *in situ* hybridisation technique. The technique involves

producing slot or dot blots of DNA from the parents and progeny, washing or blocking with DNA from the female parent before hybridising with labelled DNA from the male parent. The technique was tested on a cross between *S. officinarum* x *E. arundinaceus* from Copersucar, Brazil, and on a cross between *Saccharum* spp. x *E. giganteum* from USDA-ARS, Louisiana. Strong hybridisation was observed to the male parent and to progeny with male DNA, ie hybrids. A dilution series was also established and demonstrated that the technique would also be able to detect a 1 in 20 dilution of *Erianthus* in *Saccharum* DNA, which should enable the detection of partial hybrids, or progeny of subsequent generations of introgression programs.

4. Production of future mapping populations and verification of authenticity

Five crosses were initially made. Following evaluation of progeny numbers and segregation data for numerous traits, two populations were selected as most suitable for mapping. These populations were Q117 x 74C42 and Q96 x 78N146 (now Q173). Seventy-six progeny of the former population are currently being mapped as part of CTA024. These progeny have been screened with RAPD markers to confirm their authenticity.

5. Genetic change in tissue culture

RAFs were implemented to assess the genetic integrity of transgenic sugarcane derived from two varieties, Q155 and Q117. Three transgenics from each of these varieties were compared to the parent variety for genetic integrity and agronomic performance. This work was done in collaboration with Dr Robert Birch (Botany, UQ) who supplied the transgenic material, and Dr Mike Cox (BSES) who assessed the transgenic lines in the field. We deliberately chose transgenic lines that showed a range of agronomic performance in the field. Interestingly, we demonstrated that RAF fragments can be lost from sugarcane during the tissue culture and transformation process, but more importantly, that the loss of RAF fragments was strongly correlated with loss of agronomic performance in transgenic lines. We demonstrated that some DNA is more likely to be lost during the process of introducing new genes into sugarcane. We also used a mapping population derived from a cross involving Q117 to show that the loss of DNA appears to occur in a dispersed fashion around the genome.

5.0 IMPACT

Molecular markers techniques have been successfully adapted for use in sugarcane. A range of methods is available for use, depending on the laboratory and questions being addressed. These techniques are expected to impact significantly on sugarcane breeding programs in both the short and long term. These techniques allow varieties, elite lines and germplasm accessions to be fingerprinted, avoiding problems of mis-labellings which is especially relevant in a clonally propagated crop. Significant sections of the parental collection and germplasm collection have been screened and diversity estimates calculated. This information is providing information on taxonomic relationships within this tribe and on levels of diversity within and between genera. Geographical areas of increased diversity have also been identified for future collecting trips. For the future, the potential of diversity to predict performance is being investigated. No strong correlation between diversity and

performance was identified in the current project, but more work is being done with a larger data set in an attempt to maximise the correlation.

Many techniques have been developed which will greatly increase the throughput of markers in breeding programs. Such techniques include PCR tests on intact tissue, 5S DNA and *Erianthus* primers for detecting *Erianthus* introgression.

Our discovery of loss of DNA during tissue culture and transformation, and the correlation with collateral damage is potentially a very significant one. Certainly, it is of relevance to the genetic engineering of sugarcane, but it may also be of relevance to traditional sugarcane improvement. Based on the results, a diagnostic test can be efficiently implemented to determine the components of the transformation protocol that are responsible for somaclonal variation. It may also represent an avenue for understanding the molecular basis of, and to prevention of, somaclonal variation. In the context of traditional sugarcane improvement, the discovery opens up the possibility of deletion mapping of important traits and genes in sugarcane.

6.0 PROJECT TECHNOLOGY

A provisional patent has been filed to protect the intellectual property associated with the diagnostic test for somaclonal variation.

7.0 TECHNICAL SUMMARY

See above.

8.0 RECOMMENDATIONS

Marker assisted selection needs to be fully assessed in sugarcane. Mapping populations need to be characterised for important traits to the Australian sugarcane industry.

The information content of the sorghum sb1-10 SSR marker when applied to sugarcane indicates that SSR markers will be very informative in sugarcane.

The DNA diagnostic test for somaclonal variation should be tested on a wider range of transgenic lines of sugarcane and also implemented to determine the components of the transformation protocol that are responsible for somaclonal variation.

9.0 PUBLICATIONS

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APPENDIX 1

MOLECULAR MARKER TECHNOLOGIES

i) PCR on intact leaf tissue

The template for the PCR was alkali-treated intact leaf tissue. The tissue was sampled and prepared as described by Klimyuk *et al.* (1993, Plant Journal 3, 493-494.), except that B-mercaptoethanol was included in the alkali solution (Carroll *et al.*, 1995, Genetics 139, 407-420.).

ii) AFLPs

AFLPs were performed as described by Vos *et al.* (1995, Nucleic Acid Res 23:4407-4414).

iii) Application of sorghum SSR markers to sugarcane

Twenty five sorghum SSR markers were tested for application to sugarcane. Purified genomic DNA and intact leaf tissue (see above) were used as template along with the sorghum SSR oligonucleotide primers. Only the primers corresponding to the sorghum sb1-10 locus amplified polymorphic fragments from sugarcane. This marker was shown to be particularly useful in the rapid identification of *S. officinarum*/*S. spontaneum* hybrids and genetic fingerprinting of sugarcane.

iv) RAFs (radio-labelled amplification fragments) - a high resolution molecular technology for genetic fingerprinting and application to plant breeding

We have developed a PCR-based molecular marker technology called RAFs that is derived from the RAPDs and DAFs technologies. RAPDs (randomly amplified polymorphic DNAs) utilises short random primers to amplify molecular markers that are detected by agarose gel electrophoresis followed by ethidium bromide staining. DAFs (DNA amplification fingerprinting) followed and it also uses short random primers to amplify molecular markers, but the difference is that higher primer concentrations are used at higher annealing temperatures in the PCR, and the products are detected on small silver-stained polyacrylamide gels. Our RAF technology uses the PCR protocol to DAFs, except that the PCR products are radio-labelled with ^{33}P . The detection is different and is based on polyacrylamide electrophoresis on sequencing gels followed by auto-radiography. The advantage of DAFs and RAFs over RAPDs is reliability. The advantage of RAFs over DAFs is that more markers can be simultaneously monitored, and intact leaf tissue can be used as template in the PCR. The RAF technology has been used in genetic fingerprinting, and in the assessment of the importance of genetic diversity and the feasibility of marker assisted selection in sugarcane. It was also used to demonstrate loss of DNA in transgenic sugarcane and its correlation with collateral damage. The detailed protocol has not been published at this stage but can be obtained from B Carroll.

v) RFLPs

All techniques relevant to RFLPs were as described in the Laboratory Protocols Manual developed by CIMMYT (Hoisington, D A 1992. Laboratory protocols. CIMMYT Applied Molecular Genetics Laboratory. Mexica, D F: CIMMYT).

vi) RAPDs

DNA was isolated as described by Hoisington (1992). The RAPD protocol was as described by Tao *et al.*, 1993, *Theor Appl. Genet.* 86:679-688.

vii) Genomic Slot Blots

Genomic Slot Blots were as described in Besse *et al.*, 1997, *Genome* 40:428-432.

APPENDIX 2

APPENDIX 3

APPENDIX 4

APPENDIX 5

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**FINAL REPORT - SRDC PROJECT BSCS1 - AN ASSESSMENT OF
THE APPLICATION OF DNA MARKERS TO STUDIES OF GENETIC
DIVERSITY AND MARKER ASSISTED SELECTION IN SUGARCANE**

BY

B CARROLL, L McINTYRE AND N BERDING

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