BUREAU OF SUGAR EXPERIMENT STATIONS

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FINAL REPORT – SRDC PROJECTS:

BS115 - INTROGRESSION OF ERIANTHUS GERMPLASM INTO THE SACCHARUM GENE POOL

BS139 - INVESTIGATION OF THE SACCHARUM SPONTANEUM CONTRIBUTION TO COMMERCIAL CLONES BY GENOMIC DNA IN SITU HYBRIDISATION

by

G Piperidis, A D'Hont and N Berding

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Organisations: **Bureau of Sugar Experiment Stations (BSES)**
Centre de cooperation internationale en recherche agronomique pour le developpement (CIRAD)

Principal Investigators: **Mr George Piperidis**
BSES
PO Box 86, Indooroopilly QLD 4068

Dr Angélique D'Hont
CIRAD – BIOTROP
BP 5035
34032 Montpellier
Cedex 1 FRANCE

Dr Nils Berding
BSES Meringa
PO Box 122, Gordonvale QLD 4865

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SUMMARY

The main aims of these projects were to investigate methods of introducing useful characteristics from *Erianthus arundinaceus* (Retz.) Jeswiet into the *Saccharum* gene pool, and to gain a better understanding of the genetic processes involved in interspecific and intergeneric introgression processes using molecular and cytogenetic techniques.

Several methods were investigated to overcome the barriers to hybridisation between commercial clones and *E. arundinaceus*, including chemical treatments, improving the longevity of flowering *E. arundinaceus* stalks, and using related 'bridging' species.

The use of a genetic bridge was the only successful avenue for producing intergeneric hybrids, which is consistent with results from breeding programs around the world. Intergeneric hybrids were identified between *Saccharum officinarum* L. and *E. arundinaceus*, but only 20 hybrids from one family have survived. These hybrids represent the first step toward incorporation of *E. arundinaceus* germplasm into Australian core breeding programs. Hybrid identification was based on the use of a molecular diagnostic test, which allows the identification of true intergeneric hybrids at the seedling stage. This test is efficient, simple and robust, and is now used routinely at Meringa, so the benefits are immediate and direct.

The chromosome composition of seven intergeneric hybrids between *S. officinarum* and *E. arundinaceus* has been evaluated. Irregular chromosome elimination has occurred, with either one or both parental type chromosomes eliminated in each hybrid. One hybrid contained more than the expected number of *S. officinarum* chromosomes for n+n transmission, perhaps due to a significant elimination of *S. officinarum* chromosomes after transmitting its 2n complement to the hybrid. Chromosome elimination and I_1 sterility could be indicative of a greater divergence between *S. officinarum* and *E. arundinaceus* than their morphology suggests.

The chromosome composition of commercial sugarcane clones and I_1 interspecific hybrids was evaluated using genomic *in situ* hybridisation. The proportion of complete *Saccharum spontaneum* L. chromosomes in the commercial clones studied ranged from 9% to 21%, and the proportion of chromosomes resulting from interspecific genomic exchange between *S. officinarum* and *S. spontaneum* ranged from 5% to 17%. There was no definite difference in the *S. spontaneum* contribution between the tropical and subtropical/temperate clones tested.

The proportion of chromosomes resulting from genomic exchange between *S. officinarum* and *S. spontaneum* is very interesting, since, until recently, chromosome exchange between these species was considered not to occur. Of particular interest will be to determine whether these exchanges occur in early or later generations during the nobilisation process. Depending on whether this occurs early in the nobilisation process (eg I_2 and I_3 generations when more *S. spontaneum* chromosomes are present), or in later generations, strategies such as irradiation could be devised to expedite the recombination process.

In situ hybridisation analysis of two I_1 interspecific hybrids between *S. officinarum* and *S. spontaneum* unambiguously confirmed the 2n+n chromosome transmission typical of such crosses. The next important step will be to analyse the chromosome composition of confirmed progeny from crosses involving these clones and *S. officinarum*, ie a recurrent cross of the I_1 to a different *S. officinarum* female clone.

In situ hybridisation analysis also revealed an anomaly in the labelling and/or classification of a clone in the Australian sugarcane germplasm collection. IJ76-319 is listed as a *S. officinarum* clone. However, *in situ* hybridisation analysis revealed it has 100 chromosomes derived from *S. officinarum*, nine from *S. spontaneum*, and four resulting from genomic exchange between the two species. This could be the first evidence that clones with more than 80 chromosomes are not pure *S. officinarum*. Chromosome counts and genetic fingerprints of all clones in this valuable germplasm collection is essential for future studies.

1.0 BACKGROUND

Modern sugarcane breeding programs are based on germplasm derived from intercrossing the first nobilised canes produced earlier this century. Very few parents were involved in these interspecific crosses between *S. officinarum*, the 'noble' cane, and wild species, mainly *S. spontaneum* L. Sugarcane breeders recognise that the germplasm in core breeding programs has a very narrow genetic base.

The '*Saccharum* complex' is a group of closely related interbreeding genera including *Saccharum, Erianthus, Miscanthus, Narenga* and *Sclerostachya*. Belonging to this complex, *E. arundinaceus* is a species that has appealing attributes for sugarcane breeders, including excellent vigour and ratooning, adaptability to environmental stresses such as moisture deficits and excesses, and resistance to *Pachymetra* root rot. Incorporation of these desirable traits into the core breeding germplasm would have enormous benefit for the long term sustainability and profitability of the sugar industry.

Numerous attempts at crossing *E. arundinaceus* with commercial sugarcane clones have been made with no conclusive evidence of success. One of the major obstacles appears to be when morphological characters are used to select true hybrids. Additionally, Lee (1995) identified pre-fertilisation barriers to hybridisation between commercial clones and *E. arundinaceus*. The full potential of *E. arundinaceus* would not be realised without addressing these issues.

Modern sugarcane cultivars are characterised by a high ploidy level and frequent aneuploidy, with chromosome numbers ranging from 100 to 130. The ability to differentiate between *S. officinarum* and *S. spontaneum* chromosomes in interspecific hybrids and modern cultivars was recently demonstrated by D'Hont *et al*. (1996) using genomic *in situ* hybridisation (GISH). Further, the occurrence of recombination between the chromosomes of *S. officinarum* and *S. spontaneum* in cultivars was proven, contradicting previous assumptions that no recombination occurred (D'Hont *et al*., 1996).

Knowledge of the exact chromosome composition of Australian sugarcane cultivars, and of the *S. spontaneum* chromosome transmission during the nobilisation process would lead to optimising the use of *S. spontaneum* in future introgression programs.

2.0 PROJECT OBJECTIVES

2.1 BS115 - Introgression of *Erianthus* germplasm into the *Saccharum* gene pool

- Evaluate a range of methods and the use of genetic bridges to overcome barriers to hybridisation between commercial clones and *Erianthus arundinaceus*.
- Develop methods to reliably confirm intergeneric hybrids.
- Develop a more complete understanding of chromosome behaviour in sugarcane, particularly during introgression of related species.

A range of methods was tested to overcome pre-fertilisation barriers to hybridisation between commercial sugarcane clones and *E. arundinaceus*, but the use of a genetic bridge (*S. officinarum*), ie the use of wild species related to both parents, appears to be the only successful avenue. Molecular tests are now used **routinely** at Meringa to accurately identify true hybrids at the **seedling** stage. Progress has been made toward the third objective, but a more complete understanding of intergeneric introgression will be obtained from analysing second generation introgression progeny of intergeneric hybrids.

2.2 BS139 - Investigation of the *Saccharum spontaneum* contribution to commercial clones by genomic *in situ* hybridisation

- Identify the contribution of *Saccharum spontaneum* to a number of cultivars that perform well in either tropical or subtropical/temperate regions.
- Determine the level of recombination between *S. officinarum* and *S. spontaneum* chromosomes in these clones.
- Describe the *S. spontaneum* chromosome transmission during the nobilisation process.
- Assess the potential of irradiation to enhance recombination between *S. officinarum* and *S. spontaneum* in interspecific materials.

The chromosome constitution and level of recombination has been enumerated for 10 cultivars. Three I_1 interspecific hybrids also have been examined. One of these interspecific hybrids has been crossed with *S. officinarum* to generate I_2 progeny for future analysis of chromosome transmission during the nobilisation process. Of particular interest will be to determine whether these exchanges occur in early or later generations during the nobilisation process. Depending on whether this occurs early in the nobilisation process (eg I_2 and I_3 generations when more *S. spontaneum* chromosomes are present), or in later generations, strategies such as irradiation could be devised to expedite the recombination process.

3.0 METHODOLOGY

3.1 Introduction

The genetic base of sugarcane is narrow. Introgression of related germplasm into the *Saccharum* gene pool offers an opportunity to broaden this genetic base. However, a **basic** understanding of introgression processes in sugarcane is **lacking**. These projects were aimed at gaining a better understanding of intergeneric and interspecific introgression processes in sugarcane to help introduce new germplasm more efficiently and effectively into the sugarcane gene pool.

3.2 Intergeneric crosses

Many attempts to use *E. arundinaceus* in crosses involving *Saccharum* spp. hybrids have been made at BSES Meringa using clones from the 1976 ISSCT collection in Indonesia (Berding and Koike, 1980). However, until recently, no **conclusive** evidence of success has been achieved. In project BS115, several attempts were made to introduce germplasm from *E. arundinaceus* into the *Saccharum* gene pool using techniques to overcome the barriers to hybridisation observed by Lee (1995).

One of the barriers to hybridisation is the rapid deterioration, and decrease in pollen viability, of *E. arundinaceus* flowers when placed in standard Hawaiian acid breeding solution used routinely in core crossing (Lee, 1995). Marcotting of stalks and retention of plants in pots were two avenues attempted to overcome this barrier. Marcotting involves the use of plastic sleeves, filled with moist peat, covering at least three nodes of field grown plants. This stimulates nodal root growth which facilitates the uptake of nutrients and moisture when stalks are placed in the Hawaiian breeding solution. Plants also were grown and retained in pots, rather than cutting flowers, in a 1:1:1 mix of sand:peat:vermiculite with weekly fertiliser application. Thus, flowering stalks remained alive when placed in the crossing lanterns.

Lee (1995) also observed that, in intergeneric crosses with *Saccharum* spp. hybrids, *E. arundinaceus* pollen tubes failed to reach the micropyle or lost directional control, a phenomenon also observed by Heslop-Harrison *et al*. (1984) with *Zea mays* × *Pennisetum* crosses. This may be due to the different nutritional requirements of *E. arundinaceus* pollen not being met by *Saccharum* spp. hybrid pistils. To overcome this barrier, early morning application of gibberellic acid (75 mg/L) or nutrient solution (250 g/L sucrose; 1.1 g $CaCl_2.2H_2O$; 0.28 g H_3BO_3; pH 5.0), to flowers to aid pollen tube growth following pollen application was implemented. The solutions were applied as a spray onto one flower with a second flower receiving no spray used as a control.

Finally, the use of genetic bridges also was recommended as a method for overcoming the incongruity in *Saccharum* × *Erianthus* crosses (Lee, 1995). Nagatomi and Dunckelman (1980) recommended the use of wild species related to both parents as genetic bridges. In the present study, most crosses were made with *E. arundinaceus*. However, crosses also were made using pure *S. officinarum*, *S. spontaneum*, *S. robustum* Brandes and Jeswiet, *S. sinense* Roxb., *E. elephantinus* Hook. f., *E. procerus* (Roxb.) Raizada, and *E. bengalense* (Retz.) Bharadw.

A complete listing of all intergeneric crosses made during the course of this project is given in Appendices 1.1 to 1.4.

3.3 PCR identification of hybrids

3.3.1 Intergeneric hybrids

For the identification of **true** intergeneric hybrids, advantage was taken of the length polymorphism of the 5s ribosomal DNA (rDNA) spacer region between *Saccharum* spp. and *E. arundinaceus* (D'Hont *et al*., 1995). Further, small treated leaf pieces were used as the source of template DNA in the PCR reactions according to the protocol of Klimyuk *et al*. (1993). This method of template preparation is detailed in Appendix 2.

Conditions for amplification of the 5s-spacer region were essentially as described in D'Hont *et al*. (1995) with the following modifications. PCR reactions were performed in 25 µL volumes containing 0.4 µM of each primer (P1: 5'-GTGACCTCCTGCGAAGTCCT-3'; P2: 5'-CCCATCCGTGTACTACTCTC-3'), 200 µM dNTPs, 1 × PCR buffer (10mM Tris-HCl, pH 8.3; 50mM KCl; 1.5mM $MgCl_2$; 0.01% gelatin) and one unit of *Taq* polymerase. One drop of mineral oil was added to each tube. Thermal cycling conditions consisted of one 2 min cycle at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C using an Omnigene Thermalcycler (Hybaid Ltd., Middlesex, UK).

Eight µL of dye (Appendix 3) was added to each reaction mix before gel loading. Twenty µL of the amplification products were separated on 2% agarose gels in TBE buffer at 5 V/cm for 2 h, stained in ethidium bromide and photographed under UV light. Duplicate samples were tested for each clone. Each PCR run also consisted of a control reaction with no template DNA.

3.3.2 Interspecific hybrids

Toward the objective of identifying the *S. spontaneum* chromosome transmission during the nobilisation process (BS139), interspecific hybrids between *S. officinarum* and *S. spontaneum* were authenticated with molecular markers before performing GISH. Five interspecific hybrids were tested simultaneously with their putative parents (Table 1).

Table 1. Interspecific hybrids subjected to molecular marker analysis to confirm their parentage before continuing with *in situ* hybridisation analysis

Clone	*S. officinarum* female parent	*S. spontaneum* male parent (2n)
MQ62-261	Badila	US56-13-7 (80)
MQ62-262	Badila	US56-13-7 (80)
MQ72-1068	Chittan	Saigon†
MQ72-1175	Badila	SES341 (80)
MQ72-5089	Badila	SES100A (64)

† No chromosome count of Saigon has been published.

The molecular marker used for testing these clones was a PCR-based system using primers designed to amplify a microsatellite in sorghum, the sorghum Sb1-10 marker. Once again, the source of template DNA for PCR reactions was small, treated leaf pieces (Klimyuk *et al*., 1993). PCR reactions were performed in 20 µL volumes containing 0.02 µM labelled ([γ-^{33}P]ATP) forward primer (5'- GTGCCGCTTTGCTCGCA -3'), 0.1 µM reverse primer (5'- TGCTATGTTGTTTGCTTCTCCCTTCTC-3'), 125 µM dNTPs, 1 × PCR buffer (10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 50mM KCl), 0.06 µl β-mercaptoethanol, and one unit of *Taq* DNA polymerase.

The reactions were conducted in a PE-9600 thermocycler (Perkin-Elmer Corp.). Cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 10 cycles of 15 s at 94°C, 15 s at 60°C, 45 s at 72°C, followed by 25 cycles of 15 s at 94°C, 15 s at 58°C, and 45 s at 72°C. Amplification products were separated on sequencing gels. The method used for running these gels is given in Appendix 4.

3.4 Genomic *in situ* hybridisation

GISH is a powerful cytogenetic tool for examining the chromosome composition of interspecific and intergeneric hybrids. The procedures for chromosome preparations and GISH experiments were as previously described (D'Hont *et al*., 1996). A detailed summary of the procedures for chromosome preparations and GISH is given in Appendices 5 and 6, respectively. Cells were photographed on Fujicolor 400 print film.

One of the problems encountered during the course of these projects was the difficulty in obtaining good quality chromosome spreads for GISH **consistently**. This is extremely important, but difficult to achieve for a plant with so many chromosomes. Following suggestions by Dr Berding, two avenues were investigated to overcome this problem. The first was to grow the plants in a commercial hydroponics solution specifically formulated for sugarcane. Advice from John Reghenzani and David Grace on sugarcane hydroponics was invaluable. The hydroponics solution was purchased from Growth Technology in Western Australia and consisted of the following elemental concentrations:

Element	mg/L	Source
NO_3-N	209	calcium nitrate, potassium nitrate
NH_4-N	11	calcium nitrate
total N	220	
P	30.97	monopotassium phosphate (MKP)
K	280	potassium nitrate & MKP
Ca	160	calcium nitrate
Mg	50	magnesium sulfate
S	66	magnesium sulfate
Fe	3	Fe-EDTA
Mn	1	Mn-EDTA
B	0.35	sodium tetraborate
Zn	0.2	Zn-EDTA
Mo	0.05	sodium molybdate
Cu	0.15	Cu-EDTA

Four setts of a clone were grown under glasshouse conditions in 250 mm pots containing 6 L of hydroponics solution, adjusted to a pH between 5.8 and 6.0 using 4M KOH, with continual aeration. The solution was changed every seven to ten days.

The second avenue investigated to improve the frequency of obtaining good quality chromosome spreads was possible only with the hydroponically grown plants because of the greater number of roots they produced. This allowed sequential sampling of roots at half-hourly intervals. Four to six root tips were harvested from a single clone (Q141) at half-hourly intervals (from 9:00am to 3:00pm on 25/9/97 and from 9:00am to 2:00pm on 16/10/97) to identify the optimum time, or more particularly, the optimum time after sunrise, for harvesting. Each root tip was spread on a microscope slide according to the procedure (Appendix 5). The slides were then fully screened for cells suitable for GISH. Cells were rated on a scale of 1 to 3 based on the quality of the spread (1 = poor, 3 = excellent). Generally, a cell rating ≥ 2 is suitable for *in situ* hybridisation, and < 2 the cell is suitable for counting the number of chromosomes, or for testing the GISH procedure, but not of sufficient quality for GISH. The chromosomes of a suitable cell are well condensed and spread with little or no overlap.

Thus, the total number of cells rated ≥ 2 was compared for each half-hour collection. These ratings were arbitrarily chosen, and intended only for the purpose of identifying the peak of mitotic activity, at least for clone Q141.

4.0 RESULTS AND DISCUSSION

4.1 PCR identification of intergeneric hybrids

Previously, morphological characters were used to identify true hybrid progeny from intergeneric crosses using *E. arundinaceus*. This meant that putative hybrid plants had to be grown to **maturity** before a visual assessment could be made. The development of a PCR-based diagnostic test, in conjunction with a simple DNA template preparation, enables the testing of putative hybrid progeny at the **seedling** stage. This is a simple, robust and very efficient tool which is now used routinely at Meringa, the main site of BSES's cross-pollination activities, so the benefits are direct and immediate. No lengthy DNA extraction procedures are necessary, as small treated leaf pieces are used as the source of DNA in PCR reactions. Seedlings can be tested, and true hybrids identified within six weeks of germination. The advantages are obvious: non-hybrids, probably resulting from self-pollination of the female parent, or pollen contamination, can be discarded immediately after PCR results have been examined. This would eliminate the competition for survival faced by real hybrids, which are weak and non-vigorous. Early discard of non-hybrids represents considerable savings in resources, particularly time and space.

An example of the PCR results obtained with this procedure is given in Figure 1. This figure shows the unequivocal clarity of the results generated using this system. Non-hybrids do not display the male *E. arundinaceus* specific band, and can be disposed of immediately after results are obtained. True hybrids can then be managed appropriately.

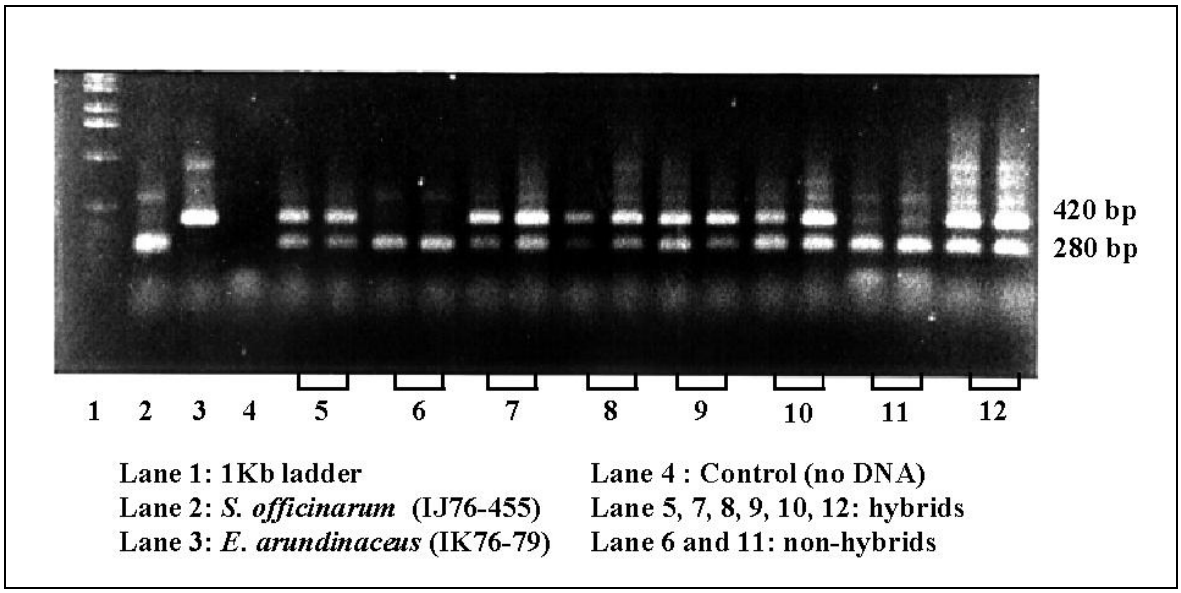

Figure 1. Intergeneric hybrid confirmation (*S. officinarum* × *E. arundinaceus*) using PCR amplification of the 5s intergenic spacer

4.2 Barriers to hybridisation

Of the range of methods investigated to overcome the barriers to hybridisation between commercial clones and *E. arundinaceus*, the use of a genetic bridge appears to be the only successful avenue. No hybrids were identified when marcotted stalks or potted plants were used in crosses. No hybrids resulted from crosses with the nutrient spray treatment. One flower treated with gibberellic acid resulted in 16 true hybrids out of 22 seedlings germinated. However, this was not significantly different from the control flower of the same cross, which resulted in 18 true hybrids from 23 seedlings germinated, suggesting that gibberellic acid also had no effect on inducing intergeneric fertilisation.

Thus, true hybrids were generated only when pure *S. officinarum* (2n=80) was used as the female parent, which is consistent with all reported results from overseas (D'Hont *et al*., 1995; Besse *et al*., 1997). *S. officinarum* can therefore be considered as a genetic bridge, and currently, the only prospective means for introducing important characters from *E. arundinaceus* to commercial clones.

4.3 Intergeneric hybrids

Nine hundred seedlings from 99 intergeneric crosses (not including crosses made in 1998) were tested using the 5s PCR system. Thirty-seven were identified as true hybrids, but only 20 have survived and currently remain under propagation at Meringa (Table 2). The surviving hybrids were from a cross between IJ76-455 (*S. officinarum*) and IK76-79 (*E. arundinaceus*) made in 1994.

Confirmed intergeneric hybrids with known parentage have been reported previously (D'Hont *et al*., 1995). These hybrids were from a cross between *S. officinarum* (BNS 3066) and *E. arundinaceus* (IK76-48). Both male parents of these hybrid populations, IK76-79 and IK76-48, were also part of a diversity study by Besse *et al*. (1997). Interestingly, IK76-79 and IK76-48 formed part of a small group separated on the diversity histogram from the rest of the *E. arundinaceus* clones studied, several of which have also been used in intergeneric hybridisation attempts at Meringa. It could be important to examine more closely these genetically diverse clones to determine if there is any significance between their diversity and intergeneric fecundity. Many other *E. arundinaceus* clones exist in the germplasm collection at Meringa that were not part of the diversity study of Besse *et al*. (1997). It may also be important to characterise these clones with molecular markers to determine if any of them belong to the same group as IK76-48 and IK76-79, and should therefore be used preferentially in intergeneric crosses.

Table 2. Intergeneric crosses from which true hybrids were identified

Female parent	Male parent	No. of seedlings	No. of hybrids	No. of survivors
IJ76-455	IK76-79	45	34	20
IJ76-455	IK76-22	33	1	0
NG57-16	IS76-172	19	2	0

The chromosome constitution of seven of the hybrids developed in this project has been evaluated using GISH (Table 3, Figure 2). None of them appeared to have the full complement of 70 chromosomes (40 *S. officinarum* plus 30 *E. arundinaceus*) for n+n transmission. Between 37 and 40 *S. officinarum* and 25 and 30 *E. arundinaceus* chromosomes were observed. Interestingly, in one hybrid (95B1034) we found more than the expected 40 *S. officinarum* chromosomes, between 48 and 52, perhaps due to a significant elimination of *S. officinarum* chromosomes after transmitting its 2n complement. The other 13 hybrids have not yet been analysed.

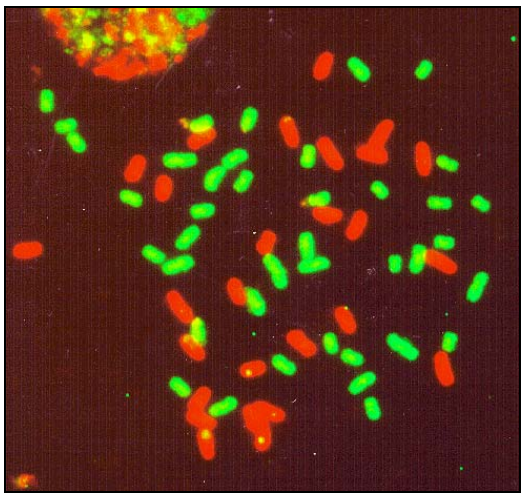

Figure 2. Genomic DNA *in situ* hybridisation on a somatic metaphase spread from an intergeneric hybrid between *S. officinarum* and *E. arundinaceus*. *S. officinarum* chromosomes are detected in green and *E. arundinaceus* chromosomes in red.

Table 3. Chromosome composition of intergeneric hybrid clones (*S. officinarum* x *E. arundinaceus*) revealed by genomic DNA *in situ* hybridisation

Intergeneric hybrid clone	2n	*S. officinarum* IJ76-455	*E. arundinaceus* IK76-79	No. of cells
95B1018	65-69	37-40	26-29	10
95B1003	64-69	38-40	26-29	13
95B1015	67	37	30	2
95B1012	65	39-40	25-26	2
95B1034	76-81	48-52	28-29	3
95B1024	63-68	38-40	25-29	4
95B1033	62-68	36-40	26-28	6

The surviving hybrids have not performed as well as expected. Many of them show undesirable growth characteristics such as aerial roots, sprawling growth habit, and poor vigour, which could be indicative of intergenomic incompatibility. This also could explain why producing intergeneric hybrids has been difficult in the past, as based on their appearance, these hybrids would not be selected for advancement, or would not be able to compete with more vigorous non-hybrids under normal cultural conditions.

That these hybrids are not as vigorous as expected should not detract from the enormous potential to be gained from incorporation of useful *E. arundinaceus* germplasm into the *Saccharum* gene pool. Many more hybrids may need to be produced before the right combination of chromosomes, or 2n+n transmission results in vigorous and more appealing clones. The main constraint in more extensive use of *E. arundinaceus* clones in crossing is the availability of flowers. This problem could be solved by initiating greater use of the photoperiod facilities at Meringa and Bundaberg to encourage and synchronise flowering with selected clones. In addition, mutagenic treatments or tissue culturing some of the existing hybrids could be utilised to induce translocations resulting in more vigorous clones and even restoration of male fertility.

One of the intergeneric hybrids produced two flowers late in the 1998 flowering season. The flowers were complete, but the anthers appeared withered, and the pollen infertile using standard tests. Consequently, they were used in two crosses with potent male clones, MQ72-5089 and 85A2652 (Appendix 1.4). The fuzz from these crosses will be germinated in March 1999, and seedlings will be subjected to PCR-based diagnostic tests to confirm their authenticity. If I_2 seedlings are identified, this will be the first evidence of successful second generation progeny derived from intergeneric introgression.

Soon after germination of seedlings from crosses made in 1995, several seedlings appeared weak and died before leaf tissue could be sampled for PCR testing. The seedlings died for no apparent reason, as other seedlings from the same family and from other families remained healthy under identical conditions. However, for three seedlings a leaf sample was secured before plant mortality. All three seedlings were identified as true intergeneric hybrids using the PCR test described previously. Presumably, the seedlings that died before leaf samples could be taken were also true hybrids. This suggests that post-germination barriers, in addition to the pre-fertilisation barriers identified by Lee (1995), are also prevalent in some crosses.

4.4 Chromosome preparations

4.4.1 Hydroponics

Hydroponically grown plants produced outstanding results compared to soil-grown plants. The most important factor was the amount of roots produced and the minimal disturbance to the plants when roots were harvested from hydroponically grown plants.

4.4.2 Sequential root tip sampling - Q141

On both occasions of the sequential root tip sampling experiments, the peak of mitotic activity was at 10:30am, followed by a smaller peak at 12:30pm (Table 4). All other collection times yielded few quality results. In a similar experiment using *E. arundinaceus*, Lee (1995) identified the peak of mitotic activity at 2:30 to 3:00pm.

These results would be strengthened by testing other clones and also by conducting the same experiments throughout the year to identify if a correlation exists between the peak of mitotic activity and the number of hours after sunrise. However these experiments are time-consuming and could not be conducted during the course of this study. The pilot study proved important because subsequent sampling was restricted to the optimum times identified, resulting in more consistent output. This is a valuable outcome for future cytological experiments in sugarcane.

1st Collection:			**2nd Collection:**		
Collection time[†]	No of cells ≥ 2[‡]	On (x) slides	Collection time	No of cells ≥ 2	On (x) slides
9:00	1	1	9:00	0	0
9:30	1	1	9:30	2	1
10:00	2	1	10:00	0	0
10:30	21	3	10:30	11	3
11:00	6	1	11:00	7	1
11:30	0	0	11:30	0	0
12:00	6	1	12:00	2	1
12:30	11	3	12:30	4	2
13:00	0	0	13:00	3	1
13:30	4	1	13:30	0	0
14:00	0	0	14:00	0	0
14:30	0	0			
15:00	0	0			

[†] First collection 25/9/97, sunrise at 6:05am;
second collection 16/10/97, sunrise at 5:49am.

[‡] See Methodology for explanation of rating.

4.5 GISH results

4.5.1 Commercial clones

The chromosome constitution of 10 commercial clones was evaluated using GISH (Table 5), some of which are reported here for the first time. The clones were chosen for their proven adaptation to either tropical or subtropical/temperate conditions based on their production record. The proportion of complete *S. spontaneum* chromosomes in these clones ranged from 9% to 21%, and the proportion of chromosomes resulting from interspecific genomic exchange between *S. officinarum* and *S. spontaneum* ranged from 5% to 17%. From these results a definite difference in the *S. spontaneum* contribution between the tropical and subtropical/temperate clones tested cannot be identified.

Table 5. Genomic *in situ* hybridisation analysis of tropical and temperate sugarcane cultivars

Clone	Chromosome composition			
	2n	*Saccharum officinarum*	*Saccharum spontaneum*	Recombinants
Tropical:				
R570	112	89	12 (10.7%)	11 (9.8%)
Q90	115	91-94	14 (12%)	6-10 (5-9%)
Q117	107-109	75-77	14-15 (13-14%)	15-19 (14-18%)
Q124	97-100	72	16-19 (16-19%)	9 (9%)
Q138	110-118	84-87	11-17 (9.3-15.5%)	11-15 (10-14%)
Temperate:				
NCo310	108-112	78-83	16-21 (14-19%)	8-15 (7-14%)
NCo376	111-113	76-78	23-24 (20-21%)	10-11 (9-10%)
Q110	101	64	16 (15.8%)	21 (20.8%)
Q141	106-108	79-82	14-15 (13-14%)	12 (11%)
Q155	107-110	77-85	11-17 (10-15.9%)	13 (12%)

The proportion of chromosomes resulting from exchange between *S. officinarum* and *S. spontaneum* is very interesting, since, until recently, chromosome exchange between these species was considered not to occur. Of particular interest will be to determine when these exchanges occur during the nobilisation process; depending on whether this occurs early in the nobilisation process (eg I_2 and I_3 generations when more *S. spontaneum* chromosomes are present), or in later generations, strategies such as irradiation could be devised to expedite the recombination process.

4.5.2 I_1 interspecific hybrids

Five I_1 interspecific hybrids were identified as potentially suitable for analysing chromosome transmission during the nobilisation process (Table 1). These hybrids were first tested with a PCR-based marker to confirm their parentage (see Figure 3). The parentage of MQ62-261, MQ72-1068 and MQ72-5089 is clearly displayed, as the corresponding male parent bands have been transferred to them. However, MQ72-1068 was no longer available at Meringa and therefore excluded from further analysis.

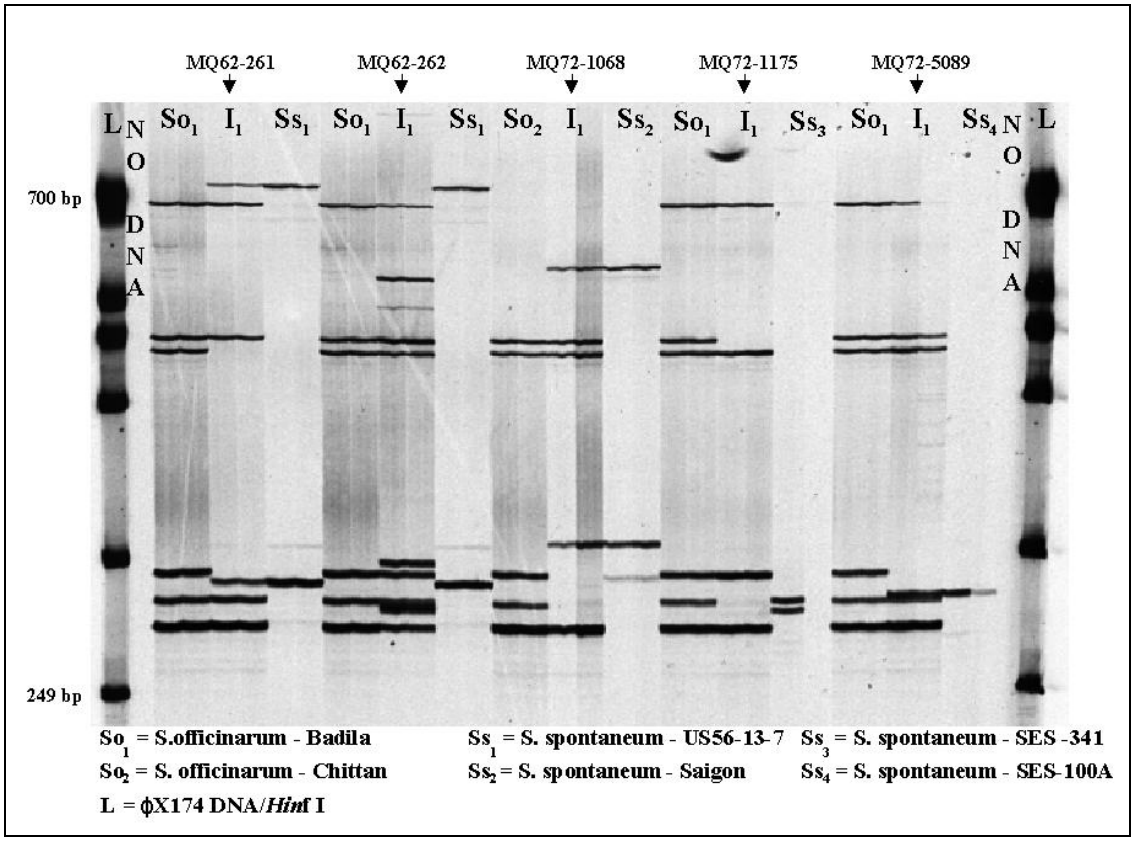

Figure 3. Interspecific hybrid confirmation (*S. officinarum* × *S. spontaneum*) using the sorghum Sb1-10 marker

MQ72-1175 did not inherit either of the two male parent bands, and may have resulted from self fertilisation of the female parent. In contrast, MQ62-262 displayed extra bands not seen in either parent. This clone was therefore assumed to have arisen from pollen contamination and was excluded from any GISH.

In situ hybridisation analysis of the interspecific hybrids, MQ62-261 and MQ72-5089, confirmed the 2n+n chromosome transmission from their respective parents (Table 6). *S. spontaneum* chromosomes were detected in MQ72-1175, which suggests that it did not arise from self fertilisation of the female parent. The number of *S. spontaneum* chromosomes found in MQ72-1175 suggest that its male parent is of the 2n = 64 type. However, Kandasami *et al*. (1983) reported **2n = 80** for SES-341. This supports the findings from the PCR analysis that SES-341 was not the pollen-donor for MQ72-1175.

MQ62-261 flowers were complete, but male sterile using routine tests. Thus, only MQ72-5089 was used in attempts to generate I_2 progeny. The next step will be to analyse confirmed progeny from crosses involving MQ72-5089 and *S. officinarum*.

Table 6. Chromosome composition of I_1 interspecific hybrid clones

Clone	Chromosome composition			Male parent 2n
	2n	*S. officinarum* Badila	*S. spontaneum*	
MQ72-5089	108-112	78-80	30-32	SES100A 2n=64†
MQ62-261	112-120	77-80	35-40	US56-13-7 2n=80‡
MQ72-1175	109-112	79-80	30-32	SES341 2n=80†

Source: † Kandasami *et al.,* 1983
‡ Roach, 1968

Seedlings from four crosses involving MQ72-5089 were planted to the field in 1997 (Table 7). These seedlings were tested with randomly amplified polymorphic DNA (RAPD) markers to confirm their hybridity (data not shown). Initial efforts were concentrated on the cross IJ76-319 × MQ72-5089 because of the greater number of seedlings available from this family (n = 45). Indeed, most of the seedlings appeared to be true hybrids between the indicated parents, only one seedling appeared to result from selfing, and another seedling from pollen contamination.

Table 7. Crosses made during the 1997 flowering season to generate I_2 progeny for *in situ* hybridisation analysis of the nobilisation process

Female parent	Male parent	No of seedlings
IJ76-319	MQ72-5089	45
NG57-59	MQ72-5089	4
MQ36-1761	MQ72-5089	4
IJ76-514	MQ72-5089	3

However, a precautionary mitotic chromosome count of IJ76-319 revealed more than the 2n=80 chromosomes characteristic of *S. officinarum* clones, even though this clone is listed as pure *S. officinarum* in the germplasm collection maintained at Meringa. *In situ* hybridisation analysis of IJ76-319 revealed 100 chromosomes derived from *S. officinarum*, nine from *S. spontaneum*, and four resulting from genomic exchange between the two species (data not shown). This is an important discovery because of the current debate among the scientific community as to whether *S. officinarum* clones

with more than 80 chromosomes are indeed pure *S. officinarum*. *In situ* hybridisation may have provided the first conclusive evidence that a clone with more than 80 chromosomes is not pure *S. officinarum*, even though it may resemble *S. officinarum* based on field evaluation.

An alternative explanation could be that the clone has been mislabelled after importation and release from quarantine. This possibility is still being investigated, although early indications are that this is not the case.

In either case, this discovery has eliminated the use of this progeny family for analysing the transmission of chromosomes during the nobilisation process, as pure *S. officinarum* is required for this purpose. Nevertheless, three clones from two other crosses also have been identified as positive hybrids using RAPDs. Chromosome counts of the female parent, NG57-59 and MQ36-1761, of these seedlings, will need to be checked before proceeding with GISH analysis. More progeny also will need to be generated.

5.0 IMPACT

Introgression of related species into the sugarcane gene pool is extensively recognised as essential for the long-term profitability and sustainability of the sugar industry. However, genetically, introgression processes in sugarcane are poorly understood. A greater understanding of these processes would lead to more directed and focussed introgression programs delivering outcomes more efficiently and effectively.

Successful incorporation of germplasm from *E. arundinaceus* into the sugarcane gene pool would have enormous benefit to the sugar industry. The intergeneric hybrids developed in project BS115 represent the first step toward this goal. More hybrids may need to be generated before the full potential is realised. These results are expected to impact significantly on continuing intergeneric introgression programs aimed at incorporating useful germplasm from *E. arundinaceus* into the *Saccharum* gene pool.

A greater knowledge of interspecific introgression processes would also lead to more efficient and effective programs aimed at utilising basic germplasm. For example, the recognition of recombination between chromosomes of *S. officinarum* and *S. spontaneum* in hybrids could lead to strategies such as irradiation to expedite this process during the nobilisation process.

6.0 RECOMMENDATIONS AND CONCLUSIONS

The use of a genetic bridge is currently the only prospective means of introducing useful characteristics from *E. arundinaceus* to commercial sugarcane clones. Consistent with confirmed results from breeding programs around the world, intergeneric hybrids were identified only from *S. officinarum* × *E. arundinaceus* crosses. These hybrids are the first step toward incorporation of *E. arundinaceus* germplasm into the core breeding program.

It is essential that:

- correct and careful management of intergeneric hybrids at Meringa is sustained to ensure the long-term survival of these **unique** clones.
- every effort is made to cross these hybrids with *S. officinarum* or *Saccharum* hybrids to produce I_2 intergeneric hybrids.
- many more crosses of the type *S. officinarum* × *E. arundinaceus* be made to generate more hybrid progeny for examination.
- greater use is made of the photoperiod facilities at Meringa and Bundaberg to allow controlled crossing of *E. arundinaceus* with selected clones.
- the genetically diverse *E. arundinaceus* clones, IK76-79 and IK76-48, and others not yet tested, be examined more closely to determine if there is any significance to the apparent relationship between their diversity and intergeneric fecundity.
- the chromosome constitution of the 13 untested intergeneric hybrids is evaluated using GISH.

It is also important that:

- mutagenic treatments or tissue culturing some of the existing hybrids be attempted to induce translocations and generation of more vigorous clones and restoration of male fertility.
- the clone labelled as IJ76-319 at Meringa is relabelled as *Saccharum* spp hybrid, **not** *S. officinarum*.
- this clone is compared at the DNA level with the IJ76-319 clone maintained in the germplasm collection at Coimbatore, India to determine if mislabelling has occurred.

In addition, it is essential that:

- molecular and cytological characterisation of all clones kept in the germplasm collection is undertaken and the results stored in an accessible database. This will require considerable resources and effort, but the long-term benefits will far outweigh the initial outlay.
- GISH analysis of all *S. officinarum* clones in the germplasm collection with more than 80 chromosomes is undertaken.
- *in situ* hybridisation analysis during the nobilisation process is completed to determine at which stage recombination between *S. officinarum* and *S. spontaneum* chromosomes occurs.
- experiments using hydroponics are continued to increase the efficiency of the *in situ* hybridisation technique.

7.0 PUBLICATIONS

At the time of writing this report, a section of the results was being prepared for publication in an appropriate scientific journal. It is envisaged that this paper will be submitted for publication before June 1999.

8.0 DIFFICULTIES

The initial BS139 proposal submitted by Drs Angelique D'Hont and Nils Berding requested a full time technician and a budget almost double that granted to achieve the stated objectives. As the accepted proposal had a reduced budget and no technical support, there should have been an amendment to the expected outputs and objectives. The initial objectives could not be fully met under these conditions.

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Appendix 1.1

Details of intergeneric crosses made during the 1994 cross-pollination season, the number of seedlings germinated, and the number of hybrids identified.

Female parent	Male parent	No. of seedlings†	No. of hybrids†
IJ76-316	IK76-79		
NCo310	IK76-79		
77N637	IK76-7		
NCo310	IK76-48		
POJ2878	IK76-48		
77N330	IK76-48		
D1135	IK76-48		
IJ76-316	IK76-48		
77A166	IK76-48		
IM76-244	IK76-48		
77A166	IK76-49		
IJ76-456	IK76-49		
NG57-141	IK76-49		
77A166	IK76-79		
77A454	IS76-163		
IJ76-456	IK76-79		
IJ76-455	IK76-79		
BN71-1309	IK76-79		
77A426	IJ76-476		
74C29	SES-309		
83C425	SES-309		
83C714	SES-309		
IJ76-455	SES-309		
NG57-59	IK76-22		
73N335	IK76-22		
NG57-61	IJ76-359		
78N303	IJ76-359		
76N2281	IS76-150		
80N320	IS76-150		
85N799	IJ76-508		
71S1171	IJ76-508		
82N460	IK76-20		
82N601	IK76-20		
83N434	IS76-163		
NG57-59	IS76-120		
IJ76-317	IK76-88		
IJ76-463	IK76-88		
D71	IK76-111		
CP78-1628	IK76-111		
IJ76-317	IJ76-174		
CP78-1628	IJ76-174		
NG57-59	IK76-88		
83N434	IJ76-395		
77N354	IJ76-395		
83N434	IS76-176		
79N599	IS76-176		
79N179	IS76-120		
85N1267	IS76-120		
80N440	IS76-62		
	Total	784	34

† Information currently not available from previous researcher

Appendix 1.2

Details of intergeneric crosses made during the 1995 cross-pollination season, the number of seedlings germinated, and the number of hybrids identified.

Female parent	Male parent	No. of seedlings	No. of hybrids
AJAX	IK76-22	1	0
BLACK INNIS	IK76-48	1	0
BLACK INNIS	IK76-79	0	0
E281	IK76-22	0	0
IJ76-325	IS76-174	1	0
IJ76-442	IS76-162	0	0
IJ76-442	IK76-62	1	0
IJ76-442	IK76-79	0	0
IJ76-455	IK76-79	13	0
IJ76-455	IK76-22	33	1
IJ76-456	IK76-62	0	0
IJ76-463	IJ76-395	13	0
IJ76-463	IS76-158	1	0
IS76-226	IK76-79	3	0
MQ28-674	IK76-79	0	0
MQ48-3605	IK76-22	0	0
MQ48-3605	IK76-79	0	0
MQ60-1949	IS76-162	2	0
MQ60-1953	IK76-22	1	0
MQ60-1953	IS76-176	0	0
NG51-95	IK76-79	0	0
NG57-16	IS76-172	19	2
NG57-191	IJ76-332	16	0
NG57-191	IS76-172	1	0
NG57-191	IMP2384	3	0
NG57-59	IJ76-395	0	0
	Total	109	3

Appendix 1.3

Details of intergeneric crosses made during the 1997 cross-pollination season, the number of seedlings germinated, and the number of hybrids identified.

Female parent	Male parent	No. of seedlings	No. of hybrids
88A2236	IK76-79	7	0
89H217	IS76-120	0	0
89H422	IK76-79	0	0
89H422	IK76-79	0	0
94B532	IJ76-394	0	0
BLACK INNIS	IS76-120	0	0
E281	IK76-111	0	0
E281	IJ76-332	0	0
IJ76-316	IK76-79	0	0
IJ76-316	IK76-79	0	0
IJ76-317	IJ76-395/IJ76-374	0	0
IJ76-442	IK76-79	0	0
IM76-244	IK76-79	0	0
IM76-244	IK76-79	0	0
MAPOU-PERLEE	IK76-79	0	0
MQ62-261	IK76-48	0	0
NG21-22	IK76-79	0	0
NG57-16	IJ76-357	0	0
NG57-191	IK76-79	0	0
NG57-59	SES311	0	0
NG57-59	IK76-79	0	0
NG57-59	IJ76-163	0	0
Q174	IK76-79	0	0
	Total	7	0

Appendix 1.4

Details of intergeneric crosses made during the 1998 cross-pollination season. These crosses will be germinated in March 1999.

Female Parent	Male Parent	No. of seedlings	No. of hybrids
95B1033	MQ72-5089		
95B1033	85A2652		
FIJI 28	IS76-174 IS76-188 IS76-193 IS76-476		
IJ76-316	IK76-79		
IJ76-514	IJ76-357 IJ76-374 IJ76-394		
NG57-191	IJ76-342 IJ76-359		
	Total		

Appendix 2

Template preparation for PCR reactions according to Klimyuk *et al*. (1995).

In 1.5 mL eppendorf tube:

1. 40 μL of solution 1, place on ice
2. Sample tissue, approximately 3 mm^2
3. Boil for 2 min, place on ice
4. Add 60 μL of solution 2
5. Boil for 2 min, place on ice. Sample thin strip of leaf (0.5 mm) for PCR or go to 6
6. Store at 4°C
7. Boil for 2 min before sampling for PCR

Solutions:

1 M NaOH: 4 g/100 mL
1 M HCl: 1 mL conc. HCl + 9.2 mL H_2O

Solution 1:

2 mL 1 M NaOH
6 mL H_2O
80 μL β-mercaptoethanol

Solution 2:

2 mL 1 M HCl
2 mL 1 M Tris
8 mL H_2O

NOTE: Take caution when boiling as the tubes can pop open.

Appendix 3

Components of loading dye used in PCR reaction mixes for loading onto agarose gels.

6x:

Ficoll	(15%)	7.5 g
0.5 M EDTA (pH 8)	(40 mM)	4 mL
Bromophenol blue	(0.1%)	50 mg
		dH_2O to 50 mls

Appendix 4

Conditions for setting up and running sequencing gels for the separation of PCR amplification products using the sorghum Sb1-10 primers.

PCR reaction products were separated on sequencing gels (Bio-Rad). Gel solutions were prepared as 150 mL of matrix consisting of 5% acrylamide, 0.25% methylene bisacryl, 7.5M Urea, in 1 x TBE. Gels were cast by first sealing the base with 30 mL gel solution containing 100 µL of 10% ammonium persulfate (APS) and 100 µL of tetra methylethylenediamine (TEMED). The remaining gel solution was mixed with 500 µL 10% APS and 100 µL TEMED before pouring.

Products from the amplification reactions were mixed with 20 µL loading dye (98% formamide, 10 mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol). The samples were heated at 90°C for 3 min and placed immediately on ice. Eight µL of each sample was loaded onto the gel and electrophoresed at 140 W for approximately 1 hour 50 min. Dephosphorylated ϕX174 DNA/*Hin*f I (Promega Corporation) was end-labelled and used as the size marker.

After separation of fragments, gels were vacuum dried for 30 min at 90°C on Whatmann 3MM paper and exposed to X-ray films (Kodak Biomax™ MR) for 24 to 48 h at room temperature.

Appendix 5

Slide preparation of root protoplast chromosomes.

1. Production of roots
Roots are preferentially harvested from plants in pots, preferably in hydroponics solution.

2. Pretreatment of roots
- Roots (0.5 cm) are harvested with fine forceps and placed directly into a solution of 0.04% 8-hydroxyquinoline for 4 h at room temperature.
- Fix in fresh 3:1 (ethanol:acetic acid) fixative for 72 h at room temperature.
- Store in 70% ethanol at 4°C.

3. Preparation of protoplasts
- Rinse in dH_2O 2 x 10 min, room temperature.
- Hydrolyse in 0.25N HCl 10 min, room temperature.
- Rinse in dH_2O, room temperature.
- Place in digestion buffer 10 min, room temperature.
- Cut the distal 1-1.5 mm of the root tip with a fine scalpel, blot away excess moisture with filter paper.
- Digest in enzyme solution for 90 to 180 min at 37°C. The length of time varies with the species and the size of the root tips.
- Rinse 2 x with dH_2O.
- Use a Pasteur pipette to carefully remove the root tips and place in dH_2O in a watch glass for 20 to 60 min at room temperature (time must be optimised).

4. Preparation of chromosome spreads
- Use a Pasteur pipette to carefully remove one root tip and place it on an acid-precleaned slide and remove the excess water with a home-made micro Pasteur pipette.
- Add 1 or 2 drops of 3:1 fixative and immediately break apart the tip and spread it with a pair of fine forceps.
- Allow to air-dry several hours and store overnight in a desiccator (37°C).

Appendix 6

Procedure for genomic *in situ* hybridisation

Hybridisation

- Dry the slide overnight at 37°C
- Prepare 3 Coplin jars : 70%, 95%, and 100% ethanol at -20°C
- Delimit the area for hybridisation on the slide
- Prepare a solution of RNAse at 37°C : 50 ml at 2µg/ml RNAse in 2SSC at 37°C (10 µL of RNAse at 10 mg/mL)
- Place the slide for 45 min in the solution of RNAse at 37°C
- Prepare a solution of 70% formamide at 70°C : place separately in a water bath at 70°C
  - 15 mL 2SSC
  - 35 mL formamide
- Prepare an incubation chamber, place at 37°C
- Mix the formamide and the 2SSC, place the slide in the solution for 2min 30s precisely
- Dehydrate by 5 min passages through the ethanol baths (70%, 95%, 100%) at -20°C
- Dry the slide vertically
- Prepare hybridisation buffer :

Formamide	15 µL
Dextran 50%	6 µL
20SSC	3 µL
SDS 20%	0.9 µL
DNA probe	150 to 250 ng
H_2O	up to 30 µL

- Denature the hybridisation buffer for 10 min at 75°C then place on ice
- Deposit 30 µL of the buffer on the slide, cover with a plastic, supple, coverslip
- Place slide in the humidified incubation chamber
- Place overnight in hybridisation oven at 37°C

Washes

- Remove the coverslip with a squirt of 2SSC
- Place the slide for 5 min in 2SSC at 42°C
- Then 2 times 5 min in a solution of 20% formamide, 0.1SSC, at 42°C
  (20 mL formamide + 5 mL 2SSC + 75 mL H_2O)
- Then 3 times 3 min in 2SSC at 42°C, leave on bench to cool for 5 min
- Finally 10 min in 2SSC at room temperature (RT)

Detection

- Place 5 min in a solution of 4SSC/Tween at RT
  (4SSC/Tween : 200 mL 20SSC + 2 mL Tween 20, up to 1 L H_2O)
- Deposit 200 µL of a 50:50 solution of Block:5% BSA-4SSC/Tween on the slide, cover with a plastic coverslip and place in the incubation chamber for 5 min at RT
- Remove the coverslip with a squirt of 4SSC/Tween

Appendix 6 cont'd

- Deposit 50 μL of a solution of 5% BSA-4SSC/Tween containing:
  5 μg/mL of Texas Red-avidine (2 mg/mL)
  and/or 20 μg/mL of Anti-Dig FITC (200 μg/mL)
- Cover with a plastic coverslip and place in the incubation chamber for 1 hr at 37°C
- Remove the coverslip with a squirt of 4SSC/Tween
- Wash 3 times 8 min in 4SSC/Tween at 37°C

Amplification

- Deposit 200 μL of 5% normal goat serum-4SSC/Tween on the slide, cover with a plastic coverslip and place in the incubation chamber for 5min at RT
- Deposit 50μl of a solution of 5% normal goat serum-4SSC/Tween containing:
  25μg/mL biotinylated anti-avidine (500μg/mL)
  and/or 10μg/mL FITC-conjugate rabbit anti-sheep
- Cover with a plastic coverslip and place in the incubation chamber for 1hr at 37°C
- Remove coverslip with a squirt of 4SSC/Tween
- Wash 3 times 8min in 4SSC/Tween at 37°C
- Repeat **detection** step for biotin-labelled probe

Counterstaining

- Deposit 100 μL of a solution of 4'6-diamidino-2-phenylindole (DAPI) at 2 μg/mL in McIlvains buffer (2 μL DAPI at 1 mg/mL in 98 μL of McIlvains buffer) or a solution of propidium iodide (PI) at 5 μg/mL in 2SSC (5 μL of PI 1 mg/mL, 95 μL 2SSC)
- Cover with a plastic coverslip and incubate for 15 to 30 min at RT
- Rinse in 4SSC/Tween then 2SSC at RT
- Mount with Vectashield mounting medium
- Observe under fluorescence microscope