REPORT FORMAT

REPORT TYPE: Annual Progress Report: [ ]

Final Report: [X]

Part 1 - Project Details

Project Title: (< 15 words) Molecular genetic markers for accelerated selection of Verticillium wilt-resistant cotton cultivars

CRDC Project Number: US33C

Part 2 - Contact Details

Admin Contact: Information Officer | | | Title (Mr/Mrs/etc) | First Name | Last Name

Organisation: The University of Sydney
(name of organisation that will be administering the funding)

Postal Address: Office of Research and Scholarships A14, The University of Sydney
PO Box
Sydney | NSW | 2006
Town | State | Postcode

(02) 9351 3392 | (02) 9351 4812 | research@reschols.usyd.edu.au
Phone | Fax | Email

Principal Researcher: Dr | Bruce | Lyon
Title (Mr/Mrs/etc) | First Name | Last Name

Organisation: The University of Sydney

Postal Address: School of Biological Sciences A12, The University of Sydney
PO Box
Sydney | NSW | 2006
Town | State | Postcode

(02) 9351 4240 | (02) 9351 4771 | brucel@bio.usyd.edu.au
Phone | Fax | Email

Project Supervisor: Dr | Bruce | Lyon
Title (Mr/Mrs/etc) | First Name | Last Name

Organisation: The University of Sydney

Postal Address: School of Biological Sciences A12, The University of Sydney
PO Box
Sydney | NSW | 2006
Town | State | Postcode

(02) 9351 4240 | (02) 9351 4771 | brucel@bio.usyd.edu.au
Phone | Fax | Email

Final Report

CRDC Project US33C

Molecular genetic markers for accelerated selection of Verticillium wilt-resistant cotton cultivars

July 1996 – June 2000

1. What was the background of the project?

Fungal diseases can inflict significant losses on cotton crops and, if not adequately controlled, could seriously affect the long-term sustainability of the cotton industry. In addition to quarantine procedures to restrict introduction of exotic pathogens, and improved cultural practices to reduce the build-up and spread of endemic pathogens, unquestionably the best defence against crop losses from pathogens is to breed resistant crop varieties. As an example, the introduction of Verticillium wilt tolerant *Gossypium hirsutum* varieties such as Sicala V-1 and Sicala V-2 has significantly reduced the impact of the fungal pathogen *Verticillium dahliae* on the Australian cotton crop.

However, epidemiological surveys that we have conducted reveal that some strains of *V. dahliae* prevalent in Australian cotton growing regions are capable of causing severe disease even on these improved *G. hirsutum* varieties. In order to maintain and enhance host plant resistance to fungal diseases, cotton breeding programs must have sufficient information regarding sources of potential resistance genes, together with effective testing and selection procedures to confirm that those genes are being efficiently incorporated into elite cultivars.

Very little is known about the genes responsible for resistance to Verticillium wilt in *Gossypium* spp. Enhanced disease resistance can be observed in some elite Australian varieties of *G. hirsutum*. However, our pathogenicity testing using a selection of *V. dahliae* isolates from NSW and Queensland reveals that much stronger and more broadly-based resistance can be found in the American *G. hirsutum* cultivar Acala Royale. Meanwhile the most effective resistance to Verticillium wilt caused by Australian isolates of the pathogen is found in the *G. barbadense* cultivar Pima S7. This result suggests that these latter varieties represent the best future source of additional or more effective genes for resistance to Verticillium wilt.

Molecular genetic techniques are increasingly being employed in attempts to identify and track the genes for valuable agronomic characteristics. Techniques that produce genetic or DNA "fingerprints" for individual plants, could play an important role in confirming that progeny plants in a breeding program receive all of the desired genes from the selected parental cultivars. Such methods, which include random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), could thus provide molecular or DNA "markers" for valuable plant traits.

Previous research from our laboratory revealed considerable genetic homogeneity among many cotton varieties, which corresponds with the established pedigrees, but also detects useable polymorphism between many varieties including those that exhibit different levels of susceptibility/resistance to Verticillium wilt. Our research has recently focused on the identification of further DNA polymorphisms between

susceptible and resistant plants with the aim of establishing associations between RAPD markers and genes imparting resistance to this disease.

This work in collaboration with Dr. Greg Constable and Mr. Peter Reid, from the Australian Cotton Research Institute, Narrabri NSW, has concentrated on examining parents and progeny of crosses designed to produce populations of plants segregating for resistance to Verticillium wilt. We have assessed F_2 plants of an intra-specific cross (Siokra 1-4 x Sicala V-1) for linkage of RAPD markers with disease resistance using over 250 random primers and a bulked DNA screening method. To date, two DNA markers have been identified that are more often associated with plants exhibiting enhanced disease resistance. We propose to begin similar analyses of a second intra-specific cross (CS 50 x Acala Royale), and the inter-specific cross *G. hirsutum* CS 50 x *G. barbadense* Pima S-7, to gain access to additional or more potent disease resistance genes.

The incidence of Verticillium wilt disease in cotton is increasing and has the potential to cost the industry millions of dollars annually in lost yield. Although little is known about the mechanism of resistance of plants towards Verticillium wilt, it is probable that crop resistance can be improved by the introduction of additional resistance genes from different sources using conventional plant breeding. Molecular genetic markers to be developed in this project will provide information on the genetic nature of resistance towards Verticillium wilt, and allow potential genes for disease resistance to be tracked during plant breeding programs.

Molecular genetic marker technology could accelerate the breeding of elite cotton cultivars with enhanced resistance to pests and diseases and improved agronomic characteristics such as increased fibre quality and yield. This project will contribute to the development of a strategic core of technical knowledge and skilled personnel for the application of molecular genetic markers in the enhancement of cotton plant breeding in Australia.

2. *What were the project objectives and to what extent were these achieved?*

(i) Conduct Verticillium wilt disease grading of F_2 progeny from the intra-specific crosses G. hirsutum *Sicala V-1 x Siokra 1-4 and Acala Royale x CS 50 and the inter-specific cross* G. barbadense *Pima S-7 x* G. hirsutum *CS 50.*

Disease grading assays for the classification of disease tolerant parental and segregating F_2 progeny plants was conducted prior to screening plants for DNA markers linked with disease resistance. For Verticillium wilt, extensive testing of parental, F_1 and F_2 plants from the crosses Sicala V-1 (resistant) x Siokra 1-4 (susceptible), Acala Royale (resistant) x CS 50 (susceptible), and Pima S-7 (resistant) x CS 50 (susceptible) was completed. These assays revealed that (a) vascular discolouration of the stem, and (b) final plant height, are reliable indicators of plant resistance or susceptibility to infection.

Disease assays have been conducted in the glasshouse on between 150-250 plants each from the F_2 populations of the three crosses. All three F_2 populations were found to exhibit continuous variation between the extremes of disease resistance and susceptibility. Nonetheless, sufficient individual F_2 plants were identified at the extremes of disease response for each population, and these were designated as disease "resistant" or "susceptible" for molecular analysis.

(ii) Identify candidate DNA markers linked with enhanced resistance to Verticillium wilt from Sicala V-1, Acala Royale and Pima S-7.

AFLP analyses have been conducted on the above parents and F_2 progeny using DNA polymorphisms that distinguish the parents. Three AFLP markers linked with disease resistance in Sicala V-1 (SV-M1, SV-M2 and SV-M3) and one with disease resistance in Acala Royale (AR-M2) have been identified. In addition, several markers linked with disease susceptibility have been detected. One such marker in Siokra 1-4 (SO-M7) is identical to the marker linked with disease resistance in Acala Royale (AR-M2), suggesting that a recombination event has occurred between the resistance locus and the DNA marker in this cultivar.

Although the identified DNA markers are linked with a high degree of confidence to either disease tolerance or susceptibility in the F_2 plants, the markers are not tightly linked with the disease response traits. However, a pair of markers has been found for Sicala V-1 that can effectively bracket the gene for disease resistance, thus providing robust evidence for association between the gene and marker(s). AFLP analysis of the F_2 progeny from Pima S-7 x CS 50 has yet to be completed due to an unavoidable delay in the disease response testing of these plants.

(iii) Confirm and map candidate AFLP markers linked with resistance to Verticillium wilt from Sicala V-1, Acala Royale and Pima S-7. If needed, isolate additional AFLP markers that bracket the resistance gene(s).

Additional AFLP genotyping of segregating F_2 progeny from the Sicala V-1 x Siokra 1-4 cross have confirmed the three AFLP markers SV-M1, SV-M2 and SV-M3 as being associated with enhanced resistance to Verticillium wilt in Sicala V-1. These DNA markers have subsequently been mapped relative to one another. SV-M1 maps to one side of the resistance locus (designated *VwR1*) at a distance of 31.5 cM, while SV-M3 and SV-M2 map on the other side with distances of 38.1 and 66 cM, respectively. Thus we now have AFLP markers from Sicala V-1 that bracket *VwR1*.

These bracketing markers can be used to identify plants that segregate for enhanced resistance to Verticillium wilt. For example, when assaying 51 segregating plants from the above cross for the presence of AFLP markers SV-M1 and SV-M3 (which map either side of the disease resistance locus in Sicala V-1) we identified seven plants that possessed both markers, and all seven were resistant to disease. We also identified 12 plants that lacked both markers, and all 12 were susceptible to disease. Thus selection for possession of both AFLP markers is diagnostic for plants with disease resistance.

We have further investigated the markers linked with enhanced resistance to Verticillium wilt in the American cultivar Acala Royale. AR-M2 has been found to be a robust marker linked with disease resistance, and we have begun the cloning and characterisation of this marker for development of a rapid DNA marker screening assay (see below). Importantly, this DNA marker is also detected in segregating progeny from Sicala V-1 x Siokra 1-4, this time linked with susceptibility to Verticillium wilt in Siokra 1-4 (SO-M7). The proximity of this marker to a disease resistance locus in both Acala Royale and Sicala V-1 suggests that the gene(s) for disease resistance maps to the same chromosomal region in both cultivars. This finding provides further evidence that the DNA markers are indeed linked with disease resistance.

(iv) Test characterised AFLP markers linked with resistance to Verticillium wilt on other cotton varieties to determine correlations in more distant germplasm.

We have established a collection of DNA samples from some 26 important cotton cultivars, including former and current CSD varieties and parental lines, and several

American *G. hirsutum* and *G. barbadense* cultivars. This plant DNA has been surveyed for the presence/absence of AFLP markers that are associated with enhanced resistance to Verticillium wilt in Sicala V-1 and Acala Royale. We have concluded that there seems to be little correlation between levels of disease resistance and possession of the candidate DNA markers in these other cotton varieties.

In retrospect, this finding is not surprising given the relatively distant linkage of the candidate markers with the resistance characters in Sicala V-1 and Acala Royale. This result confirms our current view that the current DNA markers will only be suitable for the direct selection of progeny from the original parent (eg. Sicala V-1 or Acala Royale), as more-distant relatives or progeny that have not been selected using the DNA markers may no longer possess the same combination of DNA markers and resistance gene (due to natural genetic recombination).

(v) Develop rapid molecular assays for DNA markers for enhanced resistance to Verticillium wilt from Sicala V-1, Acala Royale and Pima S-7.

The identification and confirmation of DNA markers from Sicala V-1 and Acala Royale has meant that rapid molecular assays for these markers can now be developed. We have begun by cloning and characterising the AR-M2 marker. DNA sequencing has revealed that the AFLP marker difference between Acala Royale and CS 50 results from an *Eco*RI site that is present in the Acala Royale allele but absent in the corresponding allele from CS 50. This discovery will make it possible to design a molecular test to detect plants that are homozygous or heterozygous for these DNA marker alleles and hence for the Verticillium wilt resistance locus.

3. What methodology was used, and a justification for the use of this methodology?

Levels of resistance to Verticillium wilt have been determined using the *V. dahliae* isolate 1035, which gives differential pathogenic reactions between the resistant and susceptible parental lines. These assays revealed that (a) vascular discolouration of the stem, and (b) final plant height, are reliable indicators of plant resistance or susceptibility to infection.

For bulked segregant analysis (BSA), pools of DNA were prepared from up to ten F_2 individuals that exhibited either 'resistant' or 'susceptible' symptoms following infection with the pathogen. We have also employed the technique of selective genotyping, which examines the DNA of F_2 plants individually, and thus avoids any problems that might arise from the inclusion of incorrectly graded plants in the BSA pools. By selecting plants from each extreme of the disease spectrum for each character, we would be able to reliably pinpoint markers that are associated with increased disease resistance (or susceptibility).

We have evaluated the relative usefulness of four modern molecular marker techniques in revealing genetic polymorphisms in cotton. All four techniques, namely RAPD-PCR analysis, random amplified microsatellite polymorphism (RAMPO) detection, inter-simple sequence repeat (ISSR) amplification, and amplified fragment length polymorphism (AFLP) analysis are based on PCR amplification using primer sequences that potentially target multiple loci in the genome.

For the two cultivars Acala Royale and CS 50, RAPD-PCR analysis using a total of 340 random 10-mer primers yielded an average of 6.5 bands/primer/cultivar, with 7% polymorphic between the two cultivars. RAMPO detection using six different

microsatellite probes yielded an average of 2.5 bands/primer/cultivar, with 17% being polymorphic. ISSR amplification using six different 5'-anchored primers yielded an average of 21 bands/primer/cultivar, with 14% polymorphic between the two cultivars. AFLP analysis using 19 primer combinations yielded an average of 30 bands/primer/cultivar, with 9% polymorphic between the two cultivars.

The above experimental evidence confirmed that the relatively new technique of AFLP analysis would be a very efficient method for the detection of DNA polymorphisms in cotton germplasm. Consequently, we developed an automated AFLP analysis technique that employs fluorescently-labelled markers and automatic band capture, for use in the evaluation of cotton germplasm. This technique offers efficiency gains due to the level of achievable automation, particularly for the processes of band generation and evaluation, and also because of the large number of reproducible markers which can be generated in each reaction.

The AFLP technique has subsequently been employed to identify significant DNA polymorphism within a collection of DNA samples from some 26 important cotton cultivars, including former and current CSD varieties and parental lines, and several American *G. hirsutum* and *G. barbadense* cultivars. These results indicate that the AFLP procedure is able to identify DNA polymorphism between very closely related cotton cultivars. AFLP analysis has also been successfully conducted on DNA from cotton F_2 progeny pooled on the basis of disease resistance or susceptibility. This has provided a number of AFLP markers for enhanced disease resistance that are inherently more stable and reproducible than RAPD markers.

4. Detailed results including statistical analysis of results?

The aim of this project was to develop molecular genetic markers that are linked with genes that confer Verticillium wilt resistance in elite Australian cotton germplasm. We sought to identify DNA markers linked with genes for enhanced resistance to Verticillium wilt from three diverse sources of cotton germplasm. Two *G. hirsutum* cultivars (Sicala V-1 and Acala Royale), which exhibit enhanced resistance to Verticillium wilt, and one *G. barbadense* cultivar (Pima S-7), which exhibits significant resistance to the disease.

The project has faced two major research obstacles that needed to be overcome before DNA markers for disease resistance could be identified. The first obstacle was how to reliably differentiate disease resistant and susceptible plants in a segregating plant population. The ability to assay F_2 progeny and correctly allocate a disease grading was essential if we were to subsequently sample the plants for DNA markers that were linked with disease resistance or susceptibility.

Levels of resistance to Verticillium wilt were determined using the *V. dahliae* isolate 1035, which gives differential pathogenic reactions between the resistant and susceptible parental lines. Symptoms assessed were relative plant height, extent of vascular discolouration of the stem, and the level of leaf necrosis/chlorosis.

Based on the responses to infection of the parental cultivars, vascular discolouration of the stem and final plant height were considered to be the most reliable phenotypes for the classification of F_2 individuals, while level of leaf necrosis/chlorosis was the least reliable. We continued to examine each type of disease symptom, grading the plants accordingly, and in some experiments preparing DNA pools on the basis of either vascular discolouration or final height characters. By selecting plants from each extreme

of the disease spectrum for each character, we hoped to reliably pinpoint markers that are associated with increased disease resistance (or susceptibility).

Disease assays have now been conducted in the glasshouse on between 150-250 plants each from the F_2 populations of the three crosses. All three F_2 populations were found to exhibit continuous variation between the extremes of disease resistance and susceptibility. Nonetheless, sufficient individual F_2 plants were identified at the extremes of disease response for each population, and these were designated as disease "resistant" or "susceptible" for molecular analysis.

The second major research obstacle was to identify and develop a molecular marker technology that is capable of detecting sufficient DNA polymorphism both within *G. hirsutum* and also in the inter-specific cross with *G. barbadense*. All reports to date suggest that older marker technologies such as restriction fragment length polymorphism (RFLP) would have limited success in detecting polymorphism in *G. hirsutum*, although it can be used in the inter-specific cross.

Newer marker technologies such as simple sequence repeats (SSR) are being employed by research groups in the USA, but again these markers appear to be best suited to comparisons between *G. hirsutum* and *G. barbadense*. Work by us and Dainis Rungis (CRCSCP) has confirmed this result in Australian cultivars. Moreover, finding sufficient numbers of markers to enable fine mapping in cotton using SSR technology would be prohibitively expensive.

We have instead developed two marker technologies, random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), which can more readily identify polymorphism even between cultivars of *G. hirsutum* which are known to be very closely related. For example, Sicala V-1 and Siokra 1-4 exhibited 3 and 2% polymorphism and Acala Royale and CS 50 exhibited 7 and 6% polymorphism using RAPDs and AFLPs, respectively (Table 1).

Table 1. Summary of RAPD and AFLP Analyses of Parental Cotton Cultivars

Parental cross	Sicala V-1 x Siokra 1-4	Acala Royale x CS 50
Total number of RAPD assays	340	340
Average RAPD loci per assay	9	6.5
Total RAPD loci	>2900	>2100
RAPD polymorphism (%)	96 (3%)	148 (7%)
Total number of AFLP assays	64	64
Average AFLP loci per assay	74	27
Total AFLP loci	>4700	>1700
AFLP polymorphism (%)	95 (2%)	104 (6%)

Most recently, we focused our efforts on the use of AFLPs. This is because this technology has much greater potential than RAPDs with respect to (a) the number of polymorphisms detected per assay, (b) the total number of polymorphisms that can theoretically be identified in cotton, and (c) the reproducibility of individual polymorphisms. Moreover, certain stages of the AFLP procedure can be automated with electronic data output, which can be analysed by computer with considerable time savings. Hence, in AFLPs we believe we have found a molecular marker technique capable of addressing the problem of low genetic polymorphism within *G. hirsutum*, while at the same time providing a robust and reproducible system which is equally applicable to inter-specific crosses.

We have previously detected DNA polymorphism between different cotton varieties using the RAPD-PCR technique, and for this project we initially employed the

technique to identify and characterise genetic polymorphism between Verticillium wilt-susceptible and -resistant plants which may be linked with enhanced disease resistance. F_2 plants of two intra-specific crosses (Siokra 1-4 x Sicala V-1 and CS 50 x Acala Royale) were assessed for linkage of RAPD-PCR markers with disease resistance using a total of 340 RAPD primers. For bulked segregant analysis, pools of DNA were prepared from up to ten F_2 plants that exhibited either "resistant" or "susceptible" symptoms following infection with the pathogen.

Mrs. Mona Akbari (CRDC Scholar, US34C) examined the parental cultivars and F_2 progeny from the intra-specific Sicala V-1 x Siokra 1-4 cross. A total of 340 random primers were tested on the two parents, generating over 2000 RAPD-PCR markers of which 96 are polymorphic between the two (the overall genetic difference between the cultivars was thus 3%). The polymorphic markers were screened against F_2 resistant and susceptible pools (prepared on the basis of vascular discolouration). This work identified one RAPD-PCR marker that appears to be linked with enhanced resistance in Sicala V-1 (OPG-17), and one RAPD-PCR marker that appears to be linked with susceptibility in Siokra 1-4 (OPV-18).

We conducted a similar RAPD-PCR analysis of the intra-specific Acala Royale x CS 50 cross. A total of 340 random primers were tested on the two parental cultivars, generating over 2000 RAPD-PCR markers of which 148 are polymorphic between the two (the overall genetic difference between the cultivars was thus 7%). This suggests that the parents are about 93% isogenic, and thus a little more polymorphic than are Sicala V-1 and Siokra 1-4. Screening of resistant and susceptible F_2 pools (prepared on the basis of vascular discolouration), identified two markers (OPL-08 and OPV-16) linked with enhanced disease resistance.

For the three markers associated with resistance (OPG-17, OPL-08 and OPV-16), testing of individual F_2 plants revealed imperfect linkage of the marker with the resistant trait. The majority of plants however conformed to the expected result, with most of the resistant F_2 individuals possessing the DNA markers, and most of the susceptible F_2 individuals lacking the DNA markers. This result is consistent with the markers being linked to a quantitative trait (QTL).

Subsequent intensive scrutiny of markers OPG-17, OPL-08 and OPV-16 using larger numbers of F_2 progeny failed to substantiate the previously observed results. The apparent linkage between all three markers and enhanced disease resistance that was observed in earlier experiments must result from poor reproducibility of the RAPD markers. When such RAPD markers are not reproducible, and as a consequence their appearance in amplified plant DNA samples occurs in a random manner, it becomes impossible to use them as DNA markers. Elsewhere, many research groups are now reporting similar failures in the use of RAPD technology, and many researchers no longer consider RAPD markers reliable for use in molecular plant breeding.

We have since developed the newer AFLP technology as the preferred molecular marker technique for cotton. We have applied AFLPs to the analysis of the same plant material employed in the RAPD analyses, namely the parents and F_2 progeny from the crosses Sicala V-1 x Siokra 1-4 and Acala Royale x CS 50. As before, F_2 plants were judged to be segregating for disease tolerance or susceptibility on the basis of vascular discolouration and/or final plant height. Using those primer combinations which identify polymorphisms between the parents, we have characterised a total of three AFLP markers linked with disease tolerance (SV-M1, SV-M2 and AR-M2) and one marker linked with disease susceptibility (CS-M1), (Table 2).

Table 2. Segregation of Candidate AFLP Markers in the Respective F_2 Populations

	Marker	SV-M1	SV-M2	AR-M2	CS-M1
Tolerant F_2 plants (Vascular Discolouration)	Present	14/17	12/17	13/15	5/13
	Absent	3/17	5/17	2/15	8/13
	Confidence	82%	71%	87%	61%
Linkage with VD phenotype	χ^2 (Significance)	3.73 (Y)	2.27 (Y)	2.3 (Y)	1.97 (Y)
Tolerant F_2 plants (Plant Height)	Present	11/17	5/17	10/13	3/8
	Absent	6/17	12/17	3/13	5/8
	Confidence	65%	29%	77%	63%
Linkage with PH phenotype	χ^2 (Significance)	5.2 (N)	9.2 (N)	2.6 (Y)	1.2 (Y)
Susceptible F_2 plants (Vascular & Height)	Present	8/20	4/20	6/19	19/20
	Absent	12/20	16/20	13/19	1/20
	Confidence	60%	80%	68%	95%

The presence of markers SV-M1 and SV-M2 from Sicala V-1 correlate significantly (60-82% and 71-80%, respectively) with disease tolerance based on vascular discolouration. The presence of marker AR-M2 from Acala Royale correlates significantly (68-87%) with disease tolerance based on both vascular discolouration and plant height, while marker CS-M1 from CS 50 correlates strongly (61-95%) with disease susceptibility.

Although the DNA markers are linked with a high degree of confidence to either disease tolerance or susceptibility in the F_2 plants, the markers are not tightly linked with the disease response traits. This is to be expected because if the DNA markers are not part of the actual gene which is encoding the disease response trait, there will always be a possibility of genetic recombination between the gene and the marker such that some plants will not receive both. Hence, use of these DNA markers in plant breeding would only provide a degree of confidence, rather than certainty, of linkage with the disease response trait.

One way to overcome this limitation would be to find more DNA markers that "bracket" the gene encoding the disease response trait, thereby ensuring a higher degree of confidence that, if DNA markers either side of the gene were present, then so too would be the target gene.

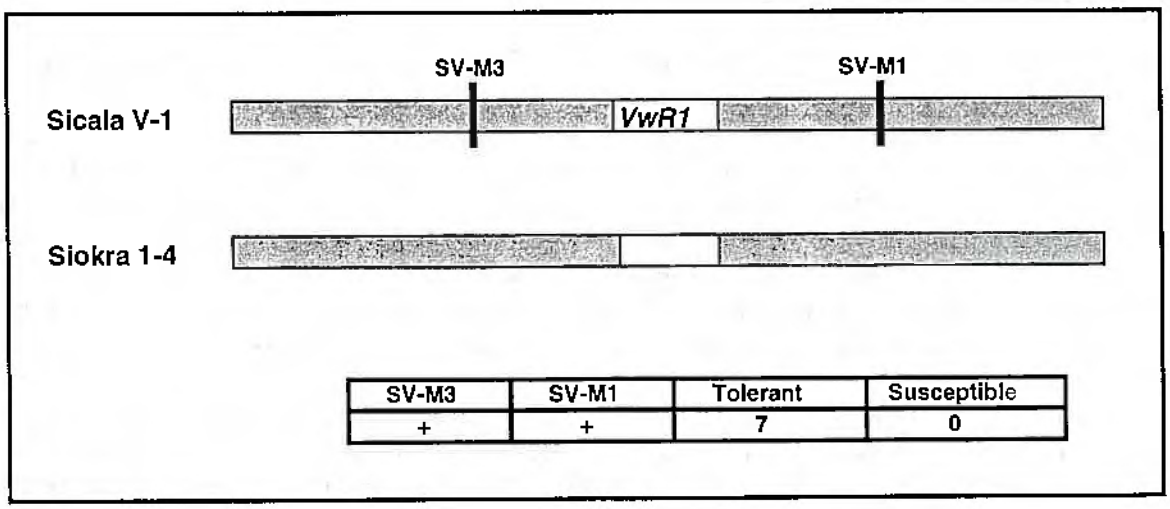

Figure 1. Diagram of AFLP markers linked with the Verticillium wilt-resistance gene *VwR1* in the Australian cotton cultivar Sicala V-1. DNA markers SV-M1 and SV-M3 bracket *VwR1* such that all progeny plants that were shown to possess these two markers are resistant to Verticillium wilt.

Enhanced resistance to Verticillium wilt in Sicala V-1 was found to be due to the action of a single major gene, designated *VWR1*. By comparing progeny plants that exhibit extremes of disease response, we have identified a handful of DNA markers that are linked with *VWR1*. The locations of the two closest DNA markers relative to the position of the putative Verticillium wilt-resistance gene are depicted in Figure 1.

These two bracketing markers could be employed to identify plants segregating for enhanced resistance to Verticillium wilt. For example, when assaying 51 F_2 plants from the above cross for the presence of AFLP markers SV-M1 and SV-M3 (which map either side of the vascular discolouration resistance locus in Sicala V-1) we identified seven plants that possessed both markers, and all seven were resistant to disease. We also identified 12 plants that lacked both markers, and all 12 were susceptible to disease.

This indicates that selection for possession of both AFLP markers is diagnostic for plants with disease resistance. The identified DNA markers are now ready to be developed for use by plant breeders to ensure that new varieties of cotton carry the *VWR1* gene for resistance to Verticillium wilt.

Testing of 26 different cotton cultivars for the presence/absence of markers AR-M2, SV-M1, SV-M2 and CS-M1 indicates poor correlation with disease resistance/susceptibility, suggesting either that this marker is not tightly linked with the trait and that recombination must have occurred in some of the cultivars, or that resistance to Verticillium wilt in these cultivars is not encoded by the particular gene(s) which we have identified in Acala Royale or Sicala V-1.

Preliminary AFLP analysis of DNA from CS 50 and Pima S-7 parental plants together with F_1 and F_2 progeny plants was conducted in readiness for molecular analysis of the new disease-graded plant population. AFLP analysis using 8 different primer combinations has shown that the number of DNA polymorphisms per primer detected between CS 50 and Pima S-7 ranges from 5 to 11 (average 7.75). These DNA polymorphisms were defined on the basis that they are present in 10/10 F_1 plants and segregate in an F_2 population of 55 plants in the expected ratio for dominant markers. Of the 62 DNA markers detected in this analysis, 21 could be associated in 9 different linkage groups, while the remaining 41 were unlinked, suggesting good dispersal over the cotton genome.

5. A discussion of the results, including an analysis of research outcomes compared with the objectives

(i) AFLPs are efficient molecular markers and show great potential for use in cotton.

We have adapted a new molecular marker technology, known as amplified fragment length polymorphism (AFLP), specifically for the identification of genetic polymorphism (plant genotyping) within *Gossypium hirsutum*. The AFLP procedure is less time-consuming, more reliable, and requires less sample DNA than any other marker technique. Most importantly, AFLPs are extremely reproducible and can be largely automated. We have chosen to use fluorescent labelling and automated signal capture to enable rapid sample processing and computer-based collation and comparison of genotype data.

The fluorescent AFLP procedure is able to detect adequate and reproducible genetic polymorphism between closely-related cultivars of *G. hirsutum* and also between *G. hirsutum* and *G. barbadense*. Furthermore, there is a much wider selection of potential

PCR primers available for AFLPs than there are with other marker techniques, and each AFLP primer pair identifies a greater number of genetic loci. This means that we can sample the cotton genome much more intensively, and the construction a high-density molecular genetic linkage map of cotton becomes a possibility.

As an example, for our current work we have used 8 different *Eco*RI primers to generate 5,000 markers in a comparison of Siokra 1-4 and Sicala V-1 (of which 100 were polymorphic). If we extrapolate the AFLP results, since there are 64 possible *Eco*RI primers, they would give rise to 40,000 markers in all, of which 800 could be expected to be polymorphic between these close parents. Under similar circumstances, 2,400 markers could be expected to be polymorphic between Acala Royale and CS 50. Numerous enzymes other than *Eco*RI could also be used to sample at other points of the cotton genome, with potentially similar numbers of markers being identified.

(ii) At least two distinct genes for Verticillium wilt tolerance have been tagged by molecular markers.

Segregation and disease testing data appears to indicate that the tagged Verticillium wilt resistance genes in Sicala V-1 and Acala Royale are genetically distinct. This means that we may have identified at least two genes that can contribute to enhanced disease resistance in *G. hirsutum*. Our evidence suggests that disease resistance in these cultivars is a quantitative trait, so the identification of two genes may imply that we have tagged two major genes contributing to the trait in these cultivars. Alternatively, the tagged genes may be unique to each cultivar, suggesting that additional major genes remain to be identified. In any event, additional molecular markers linked with disease resistance need to be identified and these should be positioned on a genetic map to determine the total number of genes contributing to this trait in cotton.

(iii) Two molecular markers will be needed to bracket each gene for Verticillium wilt tolerance.

Unless molecular markers comprise part of the actual gene which is encoding the disease response trait, there will always be a small but real possibility that the marker and trait will become dissociated in plant progeny, thereby hampering the use of any marker assay in plant breeding. Our results show that none of the identified AFLP markers are tightly linked with a disease tolerance gene.

While a search for more tightly-linked AFLP markers is possible, the effort may be unproductive, and a more effective strategy may be to identify markers which, although not tightly linked, in effect flank or bracket the target gene. Hence, two markers that are shown by mapping to bracket each gene for Verticillium wilt resistance would need to be developed for incorporation into a molecular marker assay for use in plant breeding.

(iv) Verticillium wilt resistance loci should be placed on the molecular genetic map of cotton.

Additional AFLP markers will need to be identified and the linkage of all markers with the targeted gene(s) further characterised by genetic mapping. This will make it easier to develop molecular assays that would allow plant breeders to rapidly test for the presence of DNA markers and hence for tolerance to Verticillium wilt in plant progeny.

The use of AFLP technology now makes it possible to generate a detailed molecular genetic linkage map of *G. hirsutum* onto which the genes for tolerance to Verticillium wilt could be mapped. The isolation of additional AFLP markers and the placement of these markers on a genetic map of cotton would enable each of the genes associated

with disease tolerance to be distinguished and flanked (bracket) with individual molecular markers. The bracketing of each disease tolerance gene with at least two molecular markers would be the best way to ensure a high level of confidence during selection for the trait.

AFLP technology can also be employed to detect the more abundant polymorphism that exists between *G. hirsutum* and *G. barbadense*. We still plan to apply the disease and molecular marker analyses outlined above to the F_2 progeny of the cross *G. hirsutum* CS 50 x *G. barbadense* Pima S-7. From this work we expect to identify AFLP markers linked with at least one more disease tolerance gene, possibly this time for a gene which encodes the more vigorous resistance towards Verticillium wilt exhibited by *G. babadense*. This gene could also be bracketed with molecular markers for use in a plant breeding assay if the AFLP markers were placed on a cotton genetic map.

(v) Development of a high density molecular map of cotton is now possible and should proceed with Australian involvement to maximise benefits to the local industry.

Molecular marker data from the crosses of cotton cultivars that we have been analysing, particularly CS 50 x Acala Royale and CS 50 x Pima S-7, would enable the construction of a high density genetic linkage map of Acala Royale x CS 50 x Pima S-7. This map would thus combine molecular marker data originating from both USA and Australian *G. hirsutum* cultivars with markers from *G. barbadense*.

Such a map could be integrated with inter-specific maps of cotton that are currently being made elsewhere using a range of molecular markers including RFLPs, RAPDs, AFLPs and SSRs. The development of the composite map would provide Australian researchers with access to information on the genetic locations of other desirable agronomic traits, including fibre traits such as strength, length and fineness which are derived from *G. barbadense* and are the focus of much recent research in the USA.

6. An assessment of the likely impact of the results and conclusions of the Research project for the cotton industry and, where possible, a statement of the costs and potential benefits to the Australian cotton industry and future research needs

Our research has employed molecular genetic markers to identify linkage with gene(s) for enhanced resistance to Verticillium wilt disease in cotton. For efficient utilisation in plant breeding programs, DNA markers need to be converted into stable markers that can be easily assayed. This process, currently under way for the candidate DNA markers from Sicala V-1 and Acala Royale, involves the cloning and sequencing of the candidate marker DNA, and the design of PCR primers that allow the allele-specific amplification of that DNA. To further simplify the assay, small samples of tissue would ideally be assayed soon after plant germination without affecting plant viability, and signal molecules such as fluorescent dyes, which can be detected in a fully automated manner, would be used to indicate the presence of the marker.

Molecular markers offer the potential for the accelerated breeding of cotton cultivars with more complex gene combinations, and would reduce much of the need for trait testing during plant selection. This is particularly important during the development of cultivars with enhanced resistance to biotic diseases such as Verticillium and Fusarium wilts. The utilisation of molecular markers linked with different genes for disease resistance could enable plant breeders to pyramid several combinations of these genes, thereby producing cultivars with higher disease resistance and forestalling the appearance of more virulent pathogens.

In addition to achieving a number of research objectives, the work reported here has fostered the development of a strategic core of both technical knowledge and skilled personnel for the application of molecular genetic markers in the enhancement of cotton plant breeding in Australia.

DNA marker technology holds much promise for the accelerated breeding of elite cotton cultivars not only with enhanced resistance to diseases and pests, but also with improved agronomic characteristics such as more efficient water use, increased yield and improved fibre quality. Now that the appropriate marker technologies are available, it is considered essential to develop an internationally-compatible molecular genetic map based on Australian cotton germplasm. Molecular markers could then be fully harnessed to access the superior and novel germplasm of both Australian and other cultivars.

7. A description of the project technology (e.g commercially significant developments, patents applied for or granted, licences etc)

Not applicable.

8. A technical summary of any other information developed as a part of the Research Project including discoveries in methodology, equipment design, etc

A major obstacle to the application of molecular markers in cotton has been to identify a molecular marker technology that can detect sufficient DNA polymorphism both within *G. hirsutum* and also in the inter-specific cross with *G. barbadense*. Numerous reports suggest that although older marker technologies such as restriction fragment length polymorphism (RFLP) can be used in inter-specific crosses, they detect only limited polymorphism within *G. hirsutum*. Newer marker technologies such as simple sequence repeats (SSR) are being employed by research groups in the USA, but again these markers are clearly best suited to comparisons between *G. hirsutum* and *G. barbadense* where DNA polymorphism is relatively abundant.

We have instead applied the amplified fragment length polymorphism (AFLP) technique to identify polymorphism in *G. hirsutum*, including between cultivars that are very closely related. For example using AFLPs, Sicala V-1 and Siokra 1-4 exhibited 2% polymorphism, while Acala Royale and CS 50 displayed 6% polymorphism. We have focused on the use of AFLPs as this technology has great potential with respect to (i) the number of DNA polymorphisms detected per assay, (ii) the total number of polymorphisms that can be theoretically identified in cotton, and (iii) the reproducible nature of the polymorphisms. Moreover, the AFLP procedure can be automated with fluorescent signal acquisition and electronic data output, which can be analysed by computer with considerable savings in operator time.

AFLPs therefore represent a molecular marker technique capable of addressing the problem of low genetic polymorphism within *G. hirsutum*. The technique also provides a robust and reproducible system that is equally applicable to inter-specific crosses. Using AFLPs the cotton genome can be sampled efficiently and intensively such that the construction of a high-density molecular genetic linkage map of cotton now becomes a possibility.

9. Recommendations on the activities or the steps that may be taken to further develop, disseminate or exploit the project technology

(i) Construction of a high-density AFLP/SSR genetic linkage map of cotton.

A recent review of the CRCSCP concluded that "There appears to be a very significant opportunity for the CRC to provide leadership nationally and perhaps internationally in the development of a high density molecular map of cotton and the use of molecular markers in breeding" (Report of the 5th Year Review, Stage 1 panel, July 1998). Development of a molecular genetic map based on Australian cotton germplasm, which is also compatible with cotton maps produced elsewhere, is now considered essential if we are to fully utilise molecular markers to access the superior germplasm of both Australian and other cultivars.

It is considered important to develop genetic maps both within *G. hirsutum* and also between *G. hirsutum* and *G. barbadense*, as an inter-specific cross such as CS 50 x Pima S-7 clearly provides the potential to examine traits that are markedly distinct in the two cotton species. For example, we are already analysing Verticillium resistance in *G. barbadense* and bacterial blight resistance in *G. hirsutum* in F_2 progeny from this cross. Molecular marker data from the crosses CS 50 x Acala Royale and CS 50 x Pima S-7 would enable us to construct a genetic linkage map of Acala Royale x CS 50 x Pima S-7. This map would thus combine molecular marker data originating from Australian and American *G. hirsutum* cultivars with markers from *G. barbadense*.

SSR markers, which from our own analysis and that of Mr. Dainis Rungis (CRCSCP postgraduate project SU325) are best used in inter-specific comparisons, could be used to determine the major linkage groups (chromosomes), to which AFLP markers would be added at a higher density. A set of approximately 200 SSR primers, specifically developed for use in cotton, is commercially available from Research Genetics in the USA, and the proposed AFLP map would be anchored to the international cotton genetic map using these SSR markers.

(ii) Characterisation of genes for enhanced tolerance towards Verticillium wilt.

With the completion of this project and US34C, at least two *G. hirsutum* genes for enhanced tolerance to Verticillium wilt have been identified (one each in Sicala V-1 and Acala Royale). Potentially a third will be identified in *G. barbadense* Pima S-7. Additional molecular markers will be needed to clearly distinguish each of these genes and to place them on a genetic map of cotton. In our current work we have used just eight different *Eco*RI primers to generate 5,000 markers in the comparison of Siokra 1-4 and Sicala V-1, of which 100 are polymorphic between the parents (2% polymorphism) and two appear to be linked with tolerance to Verticillium wilt. If we extrapolate these AFLP results, since there are 64 possible *Eco*RI primers, they would be expected to give rise to 40,000 markers in all, of which 800 could be polymorphic between these parents and 16 would likely be linked with disease tolerance/susceptibility.

Furthermore, the numbers of polymorphic markers obtained when examining the more genetically diverse crosses of CS 50 x Acala Royale (6% polymorphism) and CS 50 x Pima S-7 (12% polymorphism) would be significantly more favourable. Hence, more than adequate numbers of AFLP markers could be identified to enable the generation of a primary genetic linkage map of cotton, to complete the fine-mapping of markers linked with genes associated with disease tolerance, and to define the total number of such genes detectable in the three cultivars.

(iii) Development of molecular marker assays for Verticillium wilt tolerance.

Although the AFLP technique is capable of identifying linkage between molecular markers and a trait of interest, the method is technically complex and therefore not readily applicable to mass plant progeny screening. For efficient utilisation in plant breeding programs, molecular markers should ideally be converted into stable markers that can be more readily assayed. This process generally involves the cloning and sequencing of the candidate marker DNA, and the design of PCR primers that allow the allele-specific amplification of only that DNA region.

To further simplify the assay, non-radioactive signal molecules such as fluorescent dyes, which can be detected in a fully automated manner, can be used to indicate a positive result. Small samples of tissue can be assayed prior to, or soon after germination without affecting plant viability. Candidate molecular markers for enhanced tolerance to Verticillium wilt would be selected from those AFLP markers that most closely bracket each gene associated with the trait. The molecular marker assay would ideally be released for use by plant breeders as a multiplexed genotyping test that could detect the presence of two or more markers in a single assay.

(iv) Mapping of molecular markers linked with tolerance/susceptibility to other biotic diseases.

Automated data generation means that we would be able to build a catalogue of AFLP fingerprints for members of a segregating F_2 population which could be used repeatedly to examine further traits (assayed in the F_3 or subsequent generations) that differ between the common parents. Hence, the parental and progeny plants of the three crosses analysed in this project could be surveyed for other traits of interest, the genes for which could be placed on the genetic linkage map. This might include genes for tolerance to other plant diseases such as black root rot or Alternaria leaf spot, or other characteristics of agronomic importance such as okra leaf or fibre strength, length or fineness.

Furthermore, the objectives of other projects under way in the laboratory include the isolation of molecular markers linked with enhanced tolerance to Fusarium wilt (CRCSCP postgraduate project SU324); the detection of polymorphisms associated with disease resistance gene analogues (RGAs) in Australian cotton cultivars (US43C); and the identification and characterisation of genes associated with bacterial blight resistance in cotton (US48C). Molecular markers identified in these projects may be linked with genes that confer tolerance to additional biotic diseases in cotton and would enable these genes to be mapped.

10. A list of publications arising from the research project.

Akbari M, R Kota, and BR Lyon (1996). Identification of molecular markers linked to Verticillium resistance. Poster session, 8th Australian Cotton Conference, Broadbeach, Australia.

Akbari M, and BR Lyon (1998). Molecular genetic markers for leaf shape in cotton. 38th Annual General Meeting of the Australian Society of Plant Physiologists, Adelaide, Australia.

Akbari M, R Kota, LA Becerra Lopez-Lavalle, and BR Lyon (1998). Verticillium and Fusarium wilts of cotton: Molecular genetic markers for disease resistance. pp. 577-584, Proceedings of the Ninth Australian Cotton Conference, Broadbeach, Australia.

Akbari M, and BR Lyon (1999). The use of AFLP technology for the identification of molecular markers in cotton (*Gossypium hirsutum* L.). Cotton Workshop, Plant & Animal Genome VII, San Diego, USA.

Akbari M, and BR Lyon (1999). Identification of molecular markers linked with the okra leaf shape trait in cotton (*Gossypium hirsutum* L.). Poster session, Plant & Animal Genome VII, San Diego, USA.

Akbari M, R Kota, and BR Lyon (1999). Identification of molecular markers linked with Verticillium wilt resistance in cotton (*Gossypium hirsutum* L.). Poster session, Plant & Animal Genome VII, San Diego, USA.

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Hill MK, KP Ridgway, IS Bhandal, and BR Lyon (1997). Gene expression in the cotton plant following infection by *Verticillium dahliae*. Poster session, 37th Annual General Meeting of the Australian Society of Plant Physiologists, Melbourne, Australia.

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Lyon BR, MK Hill, R Kota, and KJ Lyon (1998). Isolation and characterisation of genes associated with enhanced tolerance to phytopathogenic fungi in cotton. Poster session, Plant & Animal Genome VI, San Diego, USA.

Lyon BR (1999). DNA markers and the molecular breeding of cotton. pp. 230-236, Proceedings of the First Cotton CRC Research Conference, Narrabri, Australia.

Lyon BR (1999). DNA markers and the molecular breeding of cotton. Symposium paper, First Cotton CRC Research Conference, Narrabri, Australia.

Lyon BR (1999). DNA markers and the molecular breeding of cotton. The Australian Cottongrower 20(5): 80-83.

Lyon BR (2000). DNA markers for fungal disease resistance in cotton. Symposium paper, First Symposium on Molecular Markers in Cotton, Canberra, Australia.

Lyon BR, M Akbari, LA Becerra LopezLavalle, AE Cook, S Kailasapillai, and KJ Lyon (2000). DNA markers for resistance to fungal diseases in cotton. Proceedings of the Tenth Australian Cotton Conference, Brisbane, Australia (Submitted).

Lyon KJ, AE Cook, MK Hill, and BR Lyon (1998). Screening of cotton germplasm with molecular markers for enhanced tolerance to fungal wilt disease. pp. 585-590, Proceedings of the Ninth Australian Cotton Conference, Broadbeach, Australia.

Lyon KJ, FF Ballard, and BR Lyon (2000). Utilisation of disease resistance genes in cotton. Proceedings of the Tenth Australian Cotton Conference, Brisbane, Australia (Submitted).

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