H. 2000

January, August & Final Reports

Part 1 - Summary Project Details **REPORTS**

Please use your TAB key to complete parts1,2,4 & 5

CRDC Project Number: **US43C**

January Report:	☐	Due 29-Jan-01
August Report:	☐	Due 03-Aug-01
Final Report:	☑	Due within 3 months of project completion
Project Title:		Identification and utilisation of disease resistance genes in Australian cotton cultivars

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Part 3 – Final Report Format

The points below are to be used as a guideline when completing your final report.

1. Outline the background to the project.

When faced with attack by disease-causing organisms, plants rely on an elaborate surveillance system that detects pathogens and triggers a battery of defences to protect the host. Recent studies on the DNA of plants have uncovered an extensive collection of genes that direct this recognition of harmful organisms. These disease resistance genes (R genes) are present in hundreds to thousands of copies, and generally reside in large clusters on the plant genome. Surprisingly, all of these R genes possess regions of similar DNA sequence that encode highly-conserved protein structures essential for effective plant defence. Despite this similarity, different genes can provide resistance to pathogenic organisms as diverse as bacteria, viruses, fungi and nematodes (see our background story in the January 2001 issue of The Australian Cottongrower). In earlier work we successfully cloned a small selection of R gene-like DNA sequences, known as resistance gene analogues or RGAs, from cotton. In this project we proposed to extend the existing work, and as a result we have now targeted the two major classes of R-genes in plants (NBS-LRR and STK types). We also proposed to characterise these different genes, and have attempted to link DNA polymorphism within the genes with Verticillium disease resistance in Australian cotton cultivars.

2. List the project objectives and the extent to which these have been achieved.

(i) Complete molecular characterisation of cloned cotton resistance gene analogues

The first objective has been fully achieved and expanded upon. The cloned cotton RGAs have been completely characterised and further screening of the cotton genome for RGAs has brought the total number of cotton RGA gene families available for future utilisation to at least ten.

(ii) Verticillium wilt disease testing of segregating F_2 population from a cross between CS50 and Pima S-7

The second objective has been fully achieved. A population of 90 segregating F_2 progeny from a cross between *G hirsutum* CS50 and *G. barbadense* Pima S-7 were infected with Verticillium wilt to test the disease-response and yield data on which

plants were resistant or susceptible to disease. Leaf tissue from these plants has been stored for subsequent DNA isolation and molecular analysis.

(iii) Devise PCR-based method for the identification of DNA polymorphism in disease resistance gene analogues

The third objective has not been fulfilled to our satisfaction. This area of research received considerable effort during the second year of the project, however a suitable PCR-based method for the identification of DNA polymorphism in R genes has yet to be established in cotton.

(iv) Link DNA polymorphism with disease resistance in CS50 and Pima S-7

The fourth objective has not been achieved. This area of research is dependent upon the development of a technique suitable for detecting the low level of DNA polymorphism found in cotton. The necessary disease-tested plant material is however ready to be analysed when such a technique is established.

3. How has your research addressed the Corporations three outputs: Sustainability of natural resources, profitability and competitiveness, and/or people and communities?

The breeding of new cotton cultivars with enhanced resistance to biotic diseases is crucial to the first two of the CRDC's outputs: sustainability of resources and profitability and competitiveness. Cotton plants that possess natural genetic mechanisms for effective host resistance to plant diseases will provide sustainable and competitive yields of fibre with minimal inputs of chemicals, machinery and other resources. The cloning, characterisation and tagging of cotton R genes targeted against the organisms that cause, for example, Verticillium wilt, Fusarium wilt and Bacterial blight, would lead to a quantum leap in our ability to control these diseases. Cotton breeders could ensure that all of the necessary disease resistance genes are included in the genetic make-up of improved cotton varieties bred for Australian conditions. This project has taken the first solid steps in the process, by cloning and characterising genes (called RGAs) that are the prototypes for at least 10 families of disease resistance genes in cotton. These RGA clones can be used as tags to capture and identify the R genes responsible for resistance to a variety of cotton plant pathogens. To date, no disease resistance gene has been cloned from cotton, but possession of the RGA clones should now make this objective achievable.

4. Detail the methodology and justify the methodology used.

The principle methodology for this project involved the cloning of RGA genes from cotton, and the development of a simple PCR-based assay that could be employed to establish correlations between the Verticillium wilt disease status of a given plant and the presence/absence of DNA polymorphism within RGAs.

(i) Complete molecular characterisation of cloned cotton resistance gene analogues

We have used the PCR technique and knowledge of protein motif conservation among cloned plant disease resistance genes to clone analogous genes (known as RGAs) from cotton cultivars. Subsequently we have characterised these clones by DNA sequencing. The possession of a substantial collection of RGA families in cotton provides researchers with a great potential for detecting linkage of RGAs with various fungal, viral and bacterial diseases in cotton, since it has been shown in better characterised plant species that RGAs frequently cluster in areas on the genome that contain disease resistance genes.

(ii) Devise PCR-based method for the identification of DNA polymorphism in disease resistance gene analogues

We have attempted to devise a PCR-based method for the identification of DNA polymorphism in disease resistance gene analogues. We have explored several PCR-based strategies to search for polymorphism between cultivars. The rationale behind all techniques is to walk out of the cloned RGA region into adjoining DNA sequences that are presumably less conserved, and search for polymorphism present at restriction sites. RFLP analysis is traditionally employed for the detection of DNA polymorphism between resistant and susceptible plants. However, this type of analysis requires significant quantities of DNA because of the large genome size of cotton. Due to the parallel disease testing strategy, we had insufficient quantities of DNA from F_2 individuals on which to perform RFLP analysis. In addition, the extreme number of genes in each RGA family makes it very difficult to visualise polymorphic DNA fragments. A PCR-based strategy for detecting polymorphism that demands very little DNA was therefore considered essential.

(iii) Verticillium wilt disease testing of segregating F_2 population from a cross between CS50 and Pima S-7

We infected a population of segregating progeny from a cross between CS50 and Pima S-7 with Verticillium wilt in the glass house to test the disease-response and thereby provide resistant and susceptible plant material for molecular analysis. Disease symptoms were recorded over a 6 month period for each F_2 plant, as well as for control parental and F_1 plants, to provide for direct comparison with DNA polymorphism status of individuals. Symptoms indicative of Verticillium wilt disease were leaf necrosis, vascular discolouration in the stem and reduction in height.

(iv) Link DNA polymorphism with disease resistance in CS50 and Pima S-7

Using a disease-tested population from a cross between CS50 and Pima S-7 (see previous point), we proposed to identify DNA polymorphism within RGA genes that are present/absent in all resistant plants. Detection of a RGA polymorphism linked with Verticillium wilt resistance would assist breeders to develop cultivars with enhanced disease resistance characteristics.

5. Detail results including the statistical analysis of results.

(i) Complete molecular characterisation of cloned cotton resistance gene analogues

The cloned cotton NBS-LRR (nucleotide binding site – leucine rich repeat) resistance gene analogues available from a previous study were completely characterised by DNA sequencing. Comparative analysis of the DNA sequences revealed the presence in cotton of at least four different families of RGAs, with all showing significant homology to known disease resistance genes such as *RSP2* from *Arabidopsis* and *N* from tobacco (Figure 1).

One family of NBS-LRR-like genes (Cotton RGA 1 Family) is heavily over-represented in our collection of RGAs, suggesting either a gene family with unusually many members, or that there may have been a bias in the original PCR-amplification primers towards this particular family. A decision was made to explore the possibility of primer-bias in the hope of finding further RGA families. Based on searches of the literature and the DNA sequence database, a new set of PCR primers was produced, extending across the conserved regions found in all known resistance gene products. Using these primers for PCR amplification from the Pima S-7 cultivar,

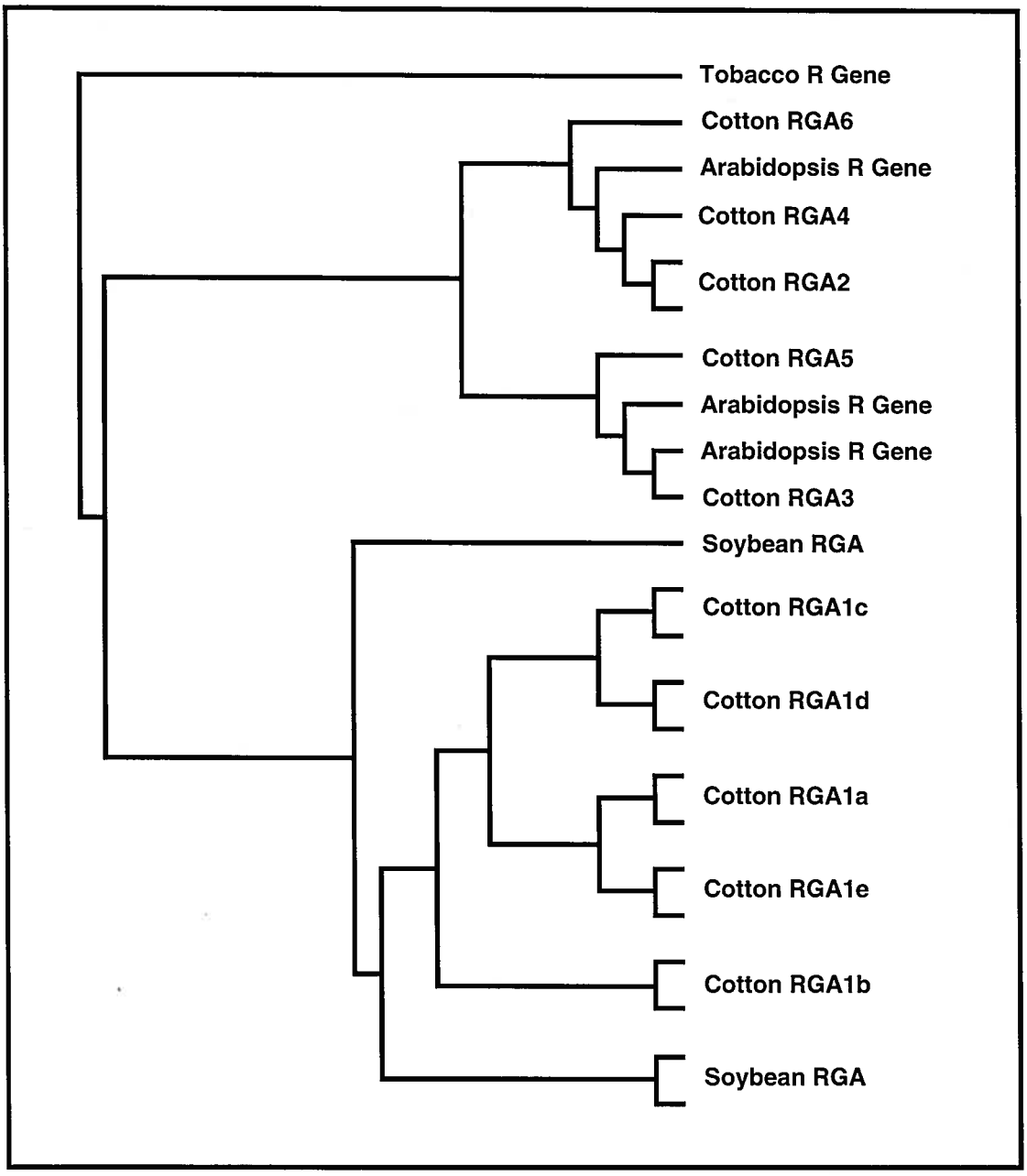

Figure 1. Family tree of cotton genes analogous to known NBS-LRR disease resistance genes (cotton RGA families 1-6). Members of cotton RGA family 1 share similarities with RGAs from soybean. Members of cotton RGA families 2-6 share similarities with disease resistance genes from *Arabidopsis*.

we successfully expanded the number of cotton RGA families. Seven hundred potential RGA clones were screened by a process of elimination and the remaining clones were characterised by DNA sequencing to reveal three new RGA families containing the appropriate conserved protein motifs. Comparison of the putative encoded RGA products allowed us to classify seven separate families including members showing 70 % or more homology, some with extensive sub-families.

This work brought the total number of cotton RGA families of the NBS-LRR type available for further analysis to seven. Homologies between families range from as low as 8 % to 43 % indicating substantial differences between some RGA families. Southern blot analysis was used to confirm the presence of the RGA families in both *G.hirsutum* and *G.barbadense*, and some polymorphic fragments could be detected between the cultivars in families with high copy number. Gene copy number was found to be very variable between families, with one family apparently single/a few copies whereas another seems to exist in hundreds of copies on the cotton genome.

From parallel work conducted as part of CRDC project US48C, we have isolated and characterised a further three families of RGAs of the STK (serine threonine kinase) type. This has brought the total number of cotton RGA gene families available for future utilisation to at least ten.

(ii) Verticillium wilt disease testing of segregating F2 population from a cross between CS50 and Pima S-7

The first attempt to complete this objective was hampered by the severe Sydney hail storm of April 1999. A population of 150 segregating progeny from a cross between CS50 and Pima S-7 had been infected with Verticillium wilt to test the disease-response and thereby provide resistant and susceptible plant material for molecular analysis. Unfortunately, the hail storm partly destroyed the glasshouse harbouring these plants, exposing them to cold and windy weather for an extended period. When the individual plants were examined for disease symptoms, the results were found to be wildly variable. It was concluded that the disease resistance results were compromised and the population was duly discarded.

This experiment was repeated during the year 2000. A population of 90 segregating F_2 progeny plants from a cross between CS50 and Pima S-7 was infected with Verticillium wilt in the glasshouse and disease symptoms recorded for each F_2 plant as well as control parental and F_1 plants over a 6 month period. Symptoms indicative of Verticillium wilt disease are leaf necrosis, vascular discolouration in the stem and reduction in plant height. Pima S-7 parental controls displayed very few symptoms, whereas CS50 parental control plants were severely affected by the disease. The F_2

plants showed a spectrum of disease symptoms, with about 25 % so severely affected that they died. This ratio of 25 % susceptible and 75 % resistant F_2 plants suggests the presence of a single dominant gene for resistance to Verticillium wilt. Cotyledons from all plants included in the disease testing were frozen in liquid nitrogen and stored at –80°C. This material is available for future extraction and examination of the DNA to search for correlations between disease resistance and a given molecular polymorphism.

(iii) Devise PCR-based method for the identification of DNA polymorphism in disease resistance gene analogues

This objective received a concerted effort during the second year of the project, however no satisfactory PCR-based method for the detection of DNA polymorphism in RGAs was established in cotton. RFLP analysis is traditionally employed for the detection of DNA polymorphism between resistant and susceptible plants. However, this type of analysis would require substantial amounts of DNA due to the large genome size of cotton, and the technique itself is quite laborious. Due to the parallel disease testing strategy, we had insufficient quantities of DNA from F_2 individuals with which to perform RFLP analysis. In addition, the extremely high copy number of genes in each RGA family makes it very difficult to visualise polymorphic DNA fragments on a Southern blot. A PCR-based strategy for detecting DNA polymorphism was therefore considered as essential as it would require very little DNA and could be conducted more efficiently.

We consequently explored a number of PCR-based strategies to search for DNA polymorphism between cotton cultivars. The rationale behind all of the employed techniques is to "walk" out of the cloned RGA region into adjoining DNA sequences that are presumably less conserved, and there to search for polymorphism present in restriction sites. The most promising technique was coined "RGA-display". This technique employs the PCR to amplify DNA segments that flank a known RGA region using RGA specific primers and primers targeting selected restriction sites. Polymorphism between cotton cultivars that exist in the targeted restriction sites would be displayed as changes in the banding pattern of the PCR products. The technique successfully displayed reproducible bands spanning up to a kilobase on each side of the cloned RGA region, however no polymorphism was detected among

the restriction sites for the several 6-bp recognition sequence enzymes employed. The technique was extended to include scanning of the amplified region by subsequent digestion with 4-bp cutters but still no polymorphism was found.

To enable a more comprehensive and better quality scanning of 4-bp restriction sites in DNA adjoining the known RGA region, we developed a technique named "gene polymorphism display". An RGA specific primer was used to produce single-stranded products extending from the known region. These products were subsequently G-tailed and amplification was performed by PCR between the gene-specific and a poly-dG primer. The amplification products were digested with 4-bp cutters, adaptors were ligated to the ends, and a second PCR reaction was performed using primers that recognise the adaptors. Finally, the PCR fragments were visualised by gel electrophoresis. We obtained a clear and reproducible banding pattern that differed substantially between cultivars, however the banding pattern also differed when different DNA preparations of the same plant were tested. Clearly, the banding pattern did not reflect the presence/absence of restriction sites only, but was affected by some unknown characteristic of the DNA preparations.

Literature searches reveal only one established method for the detection of DNA polymorphism within specific genes: Single-Nucleotide Polymorphisms (SNPs). SNPs require the sequencing of potentially large amounts of the gene of interest from both parental cultivars to detect a useful polymorphism. The polymorphism can then be detected in a PCR assay, which would discriminate between the parental DNA forms in the F2 progeny. We considered a version of this strategy. We proposed to RACE to the 5'end of specific resistance gene cDNAs. This would allow us to sequence the untranslated 5'UTR regions that should be less conserved than the coding regions searched previously. By switching our polymorphism searches to cDNA rather than genomic DNA, we would reduce the extreme complexity that results from the extended gene families, since results from our sequencing work reveal that only a small proportion of RGAs in the genome are functional and therefore expressed as cDNAs. Obviously, the Verticillium resistance gene that we hope to identify is a functional gene. A significant problem with this approach however, is that within many of the RGA families, the family members are very similar and 'gene-specific' primers based on current sequence are seldom limited to

annealing with a single RGA gene. This could lead to wrongful detection of DNA polymorphism that merely reflects differences between gene family members. A better strategy using SNPs would involve sequencing the LRR region of RGAs from BACs since DNA polymorphism in this region is likely to be more common, and also should directly reflect functional differences between resistance genes.

The overall conclusion is that the level of DNA polymorphism in cotton, especially within the region of resistance gene analogues accessible to us, seems so low that base changes within restriction sites are unlikely to identify sufficient polymorphism.

(iv) Link DNA polymorphism with disease resistance in CS50 and Pima S-7

This objective has not been achieved. A technique suitable for detecting the low level of DNA polymorphism in cotton needs to be developed. The necessary disease-tested plant material is however available and we have submitted a proposal to the CRDC for further funding to achieve this objective, after a suitable method for detection of DNA polymorphism has been established using a different strategy.

6. Discuss the results, and include an analysis of research outcomes compared with objectives.

Please see the previous section for results presentation combined with discussion.

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry and future research needs.

This project has taken the first significant steps in the process of ensuring that new cotton cultivars can be bred with stable and effective resistance to the most common forms of plant disease likely to be encountered in Australia. By cloning and characterising genes (called RGAs) that are the prototypes for at least 10 families of disease resistance genes in cotton, we have founded a molecular genetic resource that could be used repeatedly over the next decade to help localise natural cotton disease resistance genes for existing or new problem diseases. To date, no disease resistance gene has been cloned from cotton, but possession of the RGA clones should now make this objective more achievable. We recommend that the industry

look to the funding of a follow-on project proposed by our research group that seeks to exploit this resource (see section 10). This research would employ the extensive library of RGA genes analysed in this project, together with our well-characterised collections of disease resistant and susceptible cotton tissue and germplasm, to clone R genes that will provide protection against the problem diseases Fusarium wilt, Verticillium wilt and Bacterial blight. The molecular tagging of R genes for these diseases would enable plant breeders to develop elite new cotton varieties with effective disease resistance using the modern techniques of molecular plant breeding. The development of cotton varieties with disease resistance derived by natural breeding and transfer of genes within cotton would be more technically feasible and commercially desirable than relying on transgenic constructions that use genes and control mechanisms taken from foreign species.

8. Describe the project technology (eg. commercially significant developments, patents applied for or granted licenses etc).

Not applicable.

9. Provide a technical summary of any other information developed as part of the research project. Include discoveries in methodology, equipment design, etc.

We have successfully cloned RGAs from cotton by the PCR using sets of specially-designed degenerate oligonucleotide primers based on strategic regions of R-gene similarity. We have targeted two classes of R-genes, namely the NBS-LRR type and the serine threonine kinase (STK) type. By a novel process of hybridisation screening and elimination we have isolated and characterised seven different cotton RGA families exhibiting the appropriate motifs for the NBS-LRR type resistance genes, and three different cotton RGA families exhibiting the appropriate motifs for the STK type resistance genes. The methodology that we have developed for the isolation and cloning of R gene-like sequences could be applied more widely to isolate homologous genes from other *Gossypium* species, for example native Australian *Gossypium* species that harbour genes for resistance against endemic strains of Fusarium wilt. The same methods could be adapted to isolate remaining classes of R genes, which although found to be rare in other plant species, may be more prevalent in cotton.

10. State the recommendations on the activities or other steps that may be taken to further develop, disseminate, or to exploit the project technology.

A major challenge remains to determine which of the cotton RGA clones represent resistance genes, and what is the pathogen that they protect against. We recommend that genes for resistance to Fusarium wilt, Verticillium wilt and Bacterial blight should be priority targets, as these are examples of fungal and bacterial diseases of significant economic importance to the cotton industry. Cultivars of known disease response such as MCU-5 (*G. hirsutum*, resistant to Fusarium wilt), Pima S-7 (*G. barbadense*, resistant to Verticillium wilt) and CS50 (*G. hirsutum*, resistant to Bacterial blight) should be investigated. We have extensive plant genetic resources from our previous research on the genetics of disease resistance in cotton, including collections of characterised F_2 progeny plants that segregate for resistance or susceptibility to the above three diseases.

We recommend an approach comprising three major aims:

(i) The first aim would be to identify and physically map the major genomic clusters of R genes in cotton using BAC library clones. Disease resistance genes reside in large clusters in the genomes of other plant species and we expect the same to occur in cotton. Characterisation and mapping of these gene clusters would identify the hot spots in the cotton genome where active R genes are likely to be located. This would significantly decrease the amount of the genome that needs to be screened in order to localise valuable R genes.

(ii) The second aim would be to characterise single nucleotide polymorphisms (SNPs) linked with each R gene cluster. Genetic polymorphism has been shown to be rare in cotton and conventional DNA marker methods such as RFLPs, AFLPs and SSRs are relatively inefficient in detecting DNA polymorphism linked with disease resistance. DNA sequencing of BAC clones representative of the major R gene clusters in cotton would enable the design of PCR primer pairs that could amplify specific regions of high sequence variation at each cluster. These primer pairs would be employed to amplify homologous DNA fragments from a range of different cotton cultivars. DNA sequencing of the resultant fragments would enable the identification of SNPs that can be used to differentiate the cultivars.

(iii) The third aim would be to use some of the SNPs identified above as molecular probes on a selection of disease-resistant and -susceptible cotton cultivars. PCR primers/probes that could discriminate specific SNPs would be employed to amplify DNA fragments from selected parental cultivars and their F_1 and F_2 progeny that are resistant or susceptible to Fusarium wilt, Verticillium wilt or Bacterial blight. Comparison of the PCR results for different plants should enable the identification of SNPs that correlate with disease resistance or susceptibility. This technology would thus endeavour to provide an ability to tag genes that mediate resistance to the three most important biotic diseases of cotton. The molecular tagging of R genes for these diseases would enable plant breeders to develop elite new cotton varieties with effective disease resistance using modern molecular plant breeding techniques.

11. List the publications arising from the research project.

Lyon K, F Ballard, and B Lyon (2001). Plants strike back: Self-defence in the cotton field. The Australian Cottongrower, January-February, 2001.

Lyon KJ, FF Ballard, and BR Lyon (2000). Utilisation of disease resistance genes in cotton. pp. 475-478, Proceedings of the Tenth Australian Cotton Conference, Brisbane, 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. pp. 479-482, Proceedings of the Tenth Australian Cotton Conference, Brisbane, Australia.

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

Lyon BR, FF Ballard, LA Becerra Lopez-Lavalle, AE Cook, K Ho, S. Kailasapillai, and KJ Lyon (2000). Cottoning onto Pests: the Answer's in Genes. Activities for Science Week, The Cotton Store, Sydney, Australia.

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

Ballard FF, KJ Lyon, and BR Lyon (1999). Resistance gene analogue (RGA) families in cotton (*Gossypium* spp.). Poster session, 39th Annual General Meeting of the Australian Society of Plant Physiologists, Gold Coast, Australia

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 B (1999). Identification of genes for resistance to fungal wilt diseases in cotton. CRDC Fusarium Wilt Think Tank, Sydney, May 1999.

Lyon BR, AE Cook, G Cuthbert, C Faulkner, K Ho, DE Rungis, and M Tripet (1999). Genes in Your Genes. Activities for Science Week, The Cotton Store, Sydney, Australia.

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.

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

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.

Ballard F (1998). Molecular characterisation of disease resistance gene analogues in cotton. Honours Thesis, Sydney University.

Part 4 – Final Report Plain English Summary

You must submit a half to one page Plain English Summary of your research proposal that is not commercial in confidence, and that can be published on the World Wide Web. An electronic copy of the Plain English Summary must also be forwarded by e-mail (angela@crdc.org.au).

US43C: Identification and utilisation of disease resistance genes in Australian cotton cultivars

Plant diseases caused by fungal or bacterial pathogens have the potential to devastate the Australian cotton industry. It is clear that the best defence against harmful disease-causing organisms is to breed resistant cotton varieties. This project has taken the first significant steps in the process of ensuring that new cotton varieties can be bred with stable and effective resistance to all of the most common plant diseases in Australia.

By cloning and characterising genes that are the prototypes for at least ten families of disease resistance (R) genes in cotton, we have created a molecular genetic resource that could be used repeatedly to help localise natural cotton R genes for existing or new problem diseases. To date, no disease resistance gene has been cloned from cotton, but possession of the cotton R gene prototypes should now make this objective achievable. We recommend that the industry look to the funding of a follow-on project that seeks to exploit this important breakthrough in cotton genetics. This research would employ the extensive library of R gene prototypes examined in this project, together with our well-characterised collections of disease resistant and susceptible cotton plant tissue and germplasm, to clone R genes that will provide protection against the problem diseases of Fusarium wilt, Verticillium wilt and Bacterial blight.

The potential of natural cotton R genes is great, as they would provide in-built, sustainable and high-level resistance to many important diseases of cotton. Our biotechnological approach to R gene identification can be readily teamed with modern techniques of molecular plant breeding. The molecular tagging of R genes would enable plant breeders to develop and release elite new cotton varieties with effective host resistance to plant diseases. Cotton varieties with natural rather than transgenic disease resistance would be environmentally and commercially desirable.