Cotton
RESEARCH & DEVELOPMENT

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **CSP114C**

Annual Report: ☐ Due 30-September

Progress Report: ☐ Due 31-January

Final Report: ☒ Due 30-September
(or within 3 months of completion of project)

Project Title: Discovery of genes involved in the expression of cotton resistance responses to Fusarium wilt by the application of microarray technology.

Project Commencement Date: October 2000 **Project Completion Date:** October 2003

Research Program: 5 Breeding and Biotechnology

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Part 3.3 – Final Reports

1. Outline the background to the project.

Fusarium wilt disease has the potential to cause significant yield losses and the removal of some areas from cotton production. It is therefore likely to impact on the long-term sustainability of the cotton industry. Conventional breeding techniques have made some progress towards generating resistance to Fusarium wilt in the cotton plant, but breeding efforts would be enhanced by knowledge of the factors that contribute to resistance. In addition, knowledge of the factors controlling the pathogenicity of the pathogen could provide the basis for the development of novel techniques that allow minimisation of the impact of the disease. Resistance to the wilt pathogens is complex and probably controlled by many genes. This makes the task of breeding for resistance complex and difficult. In order to determine suitable approaches for improving cotton's resistance by breeding or by the use of genetic engineering, we need an improved understanding of the existing plant responses that are effective at giving some resistance to Fusarium wilt. Identification of the genes deployed by cotton during infection by the Fusarium wilt pathogen, particularly those associated with the response of more resistant species or cultivars could indicate new targets for effective breeding or for manipulation by genetic engineering. In this project, we aimed to develop tools for the large-scale study of gene expression in cotton and Fusarium, and to apply these tools to investigate gene expression in the host and the pathogen during the infection process.

Many of the techniques for the analysis of gene expression that were used when we commenced this project required gene sequence information. In this study we utilised the then novel approach of DNA microarray technology to study the expression levels of thousands of genes simultaneously with no pre-existing sequence information. Our initial aim was to set up a model system and develop the microarray tools to study gene expression. We then aimed to identify the features of gene expression associated with the responses of a moderately resistant cultivar, compared with a susceptible cultivar, and to determine if the difference in disease severity observed was due to the expression of different sets of genes, or to the expression of similar genes at different times or to a different extent.

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

The original project objectives were articulated as follows:-

Year 1. Make cDNA library from selected cotton tissue. Arrange in 96-well format. Amplify plasmid inserts, characterise size by gel electrophoresis, purify and use to print microarrays. Include appropriate negative (plant intron DNA) and positive (cDNA for known pathogen response genes) controls. Aim for 10 000 clones.

Year 2. Prepare mRNA from selected target tissues (see Table), tag with fluorescent dyes and use for screening as described in the examples given in the Table above. Perform replicate experiments to improve the confidence with which differentially expressed genes are selected. Sequence genes identified as of interest in the screening steps.

Year 3. Perform analysis of sequence information obtained, identify known genes by sequence homology and determine which would be useful for further study as potential breeding markers or by transgene-driven overexpression.

The aims behind these objectives were to identify genes in cotton and in the Fusarium wilt pathogen that showed changes during infection. We then wished to examine the changes in

gene expression in cotton that are associated with the moderately resistant cultivar, DeltaEMERALD, compared with changes occurring in the susceptible cultivar, Siokra 1-4.

3. Detail the methodology and justify the methodology used.

In order to investigate gene expression changes during infection we required a model system where we could obtain infection reproducibly in a short time period so we could generate the number of biological replicates required for robust analysis. We also needed to be able to minimise gene expression changes due to factors other than the presence or absence of infection. We developed an *in vitro* model system using deltaEMERALD seedlings that satisfied these criteria. We demonstrated infection in this system by reisolation of the pathogen from surface-sterilised tissue and observed the characteristic symptom of vascular browning in infected seedlings. For cotton there are no pre-existing, commercially available microarrays, so we generated cDNA libraries from uninfected cotton tissue and from infected tissue from a range of time points after infection. Selected clones, including appropriate positive and negative control clones, were then printed on the microarray slide. Procedures for RNA extraction, cDNA synthesis, cDNA library synthesis, microarray slide preparation, cDNA labelling, slide hybridisation and data analysis techniques were optimised for the cotton system in collaboration with other workers in the Plant Industry genomics laboratory.

Once the microarray slides had been generated, we were able to use them to compare the relative abundance of individual genes in any two samples of RNA. One of these samples was derived from infected seedlings; the other from uninfected, control seedlings. The two RNA samples were labelled with different fluorescent dyes and mixed. They were then hybridised with the cDNA molecules printed on the microarray. The relative expression level of each gene represented in the microarray was then determined by measuring the relative amounts of the two dye molecules bound to each individual spot. Analysis of differential gene expression was performed using the tRMA software suite using standard protocols. At least two biological replicates and one dye swap replicate were analysed for each pair of samples. Genes identified as differentially expressed were sequenced and subsequent assignment of putative gene function was performed using sequence homology to known genes in public databases. Differential expression of selected genes was confirmed using the alternative technique of real-time reverse transcription PCR. The involvement of certain plant hormones, such as ethylene and auxin, in the infection responses was confirmed by the analysis of hormone levels in infected and uninfected seedlings. Using these techniques, we identified the differential gene expression patterns occurring in different tissues (roots and hypocotyls) and over a range of time points (from 14 hours to 4 days) after infection.

Since one of our libraries was prepared from infected plant tissue, fungal genes that were expressed in infected tissue and not in uninfected cotton were identified by differential hybridisation to the microarray. We compared relative expression levels of fungal genes in infected plants and in saprophytically growing mycelium to identify fungal genes that may be associated with infection processes.

We found that the model system did not allow discrimination between the relatively resistant cultivar, DeltaEMERALD, and the susceptible cultivar, Siokra 1-4. We therefore developed a second infection system that allowed precise timing of infection but that minimised the impact of other environmental effects. This second system was based on stem-puncture inoculation of glasshouse-grown cotton plants. We utilised RNA prepared from stems of cotton plants of these cultivars inoculated with Fusarium wilt pathogen, or water (as control) to generate cDNA for hybridisation to the microarray to generate information about genes differentially expressed in the two cultivars after infection. Protocols for microarray hybridisation and gene expression analysis were performed as outlined above.

4. Detail and discuss the results including the statistical analysis of results.

In order to generate the microarrays for analysis of cotton gene expression, two cDNA libraries were made (one from infected, one from uninfected cotton roots and hypocotyls). 6000 clones (4000 from the Fusarium-infected cotton library, 2000 from the uninfected cotton library, a few hundred clones from a library made previously from anaerobically stressed cotton and control clones from various sources) were selected and printed on microarray slides. The quality of the resulting microarray clones was determined by analysis of a selection of clones (100 from each library). This showed that less than 5% of clones contained empty vectors, inserts were from 700 bp to 2 kbp long and the library contained largely unique sequences, showing 1% redundancy.

The identification of differentially expressed genes showed 484 clones differentially expressed in roots, with 316 of these clones showing significant matches in databases and 168 clones showing no significant matches with any sequences in the databases. These 316 clones represented 259 unique genes of which 135 were predicted to be plant genes and 126 were considered putative fungal genes. In hypocotyls, 445 clones were identified as differentially expressed with 361 of these clones showing sequence matches in databases and 84 clones showing no significant matches with any sequences in the databases. These 361 clones represented 219 unique genes of which 211 were putative plant genes and 8 were putative fungal genes.

Overall analysis of the gene expression changes occurring over time and in specific tissues revealed different gene expression profile changes in cotton root and hypocotyl tissues. In hypocotyl tissues infected with Fusarium wilt pathogen, increased expression of defence-related genes was observed, while few changes in the expression levels of defence-related genes were found in infected root tissues. In infected roots, more plant genes were repressed than were induced, especially at the earlier stages of infection. While many known cotton defence responses were identified, including induction of pathogenesis-related genes and gossypol biosynthesis genes, potential new defence responses were also identified, such as the biosynthesis of lignans. Many of the stress-related gene responses were common to both tissues. Gene expression results implicated the phytohormones ethylene and auxin in the disease process. Biochemical analysis of hormone level changes supported this observation. Perhaps the most significant novel observation made in this study was the identification of repression of genes for drought responsive proteins such as aquaporins in both roots and hypocotyls. This response may be specific to vascular wilt diseases. In the comparison of gene expression changes after infection in the moderately resistant deltaEMERALD and suceptible Siokra 1-4, the main difference observed between the two cultivars was the more extensive repression of aquaporin genes in infected Siokra 1-4 stems. While our experiments have not been able to ascertain whether this repression is a cause of susceptibility or a consequence of more extensive infection, they have identified aquaporins as important in the development of Fusarium wilt disease and therefore as potential targets for further investigation.

In the analysis of fungal gene expression over time in infected and uninfected cotton root and hypocotyl tissues we identified 126 Fusarium wilt pathogen genes. Assignment of putative gene function on the basis of sequence homology showed that many of the fungal genes expressed in infected roots were associated with metabolism, particularly energy metabolism. Comparison of Fov gene expression levels in infected roots and in saprophytically grown mycelium showed that some genes, such as a homologue of a gene, *tef1,* encoding for a putative translation elongation factor 1 alpha, were highly expressed both during infection and during saprophytic growth. We identified genes with higher relative expression levels in

infected tissue than in saprophytic mycelium that are potentially specific for *in planta* fungal processes. One such gene is a homologues of the *AtsC* gene, shown to have a role in virulence in *Agrobacterium tumefaciens*. In inoculated cotton stems, higher relative levels of *AtsC* expression were associated with inoculation using a pathogenic Fusarium isolate than when a non-pathogenic isolate was used for both Siokra 1-4 and deltaEMERALD. One particularly interesting observation was the detection of high levels of expression of the retrotransposon *foxy.* Expression of *foxy* and other retrotransposon genes could be providing a mechanism for genetic variation in Fov. Curt Brubaker and coworkers, following up on this observation, have shown that *foxy* is moving in field population of Fusarium wilt pathogen (unpublished observations).

One of the limitations of the microarray experiments performed to date is that we were attempting to associate gene expression changes with resistance using commercial cotton cultivars where limited resistance is expressed. We therefore collaborated with Curt Brubaker (CSP120C) in the assessment of wild cotton species as potential sources of resistant cotton. Promising results have been obtained with some *G. sturtianum* accessions that could be used for further comparisons of gene expression patterns in susceptible and resistant cottons.

5. Provide a conclusion as to research outcomes compared with objectives. What are the "take home messages"?

We have developed effective microarray and model infection systems for the analysis of gene expression in Fusarium-infected cotton. This is a resource of potential use to the cotton industry in the identification of potential targets for generation of improved resistance to Fusarium wilt.

In infected seedlings, gene expression changes in roots and hypocotyls appear to be different.

Repression of gene expression, particularly repression of genes for water-regulating proteins, such as aquaporins, is an important novel observation. This repression appears to be associated with susceptibility. This identifies aquaporins as potentially important in the development of wilt symptoms.

In the Fusarium wilt pathogen, expression of a homologue of *AtsC* may be important for pathogenicity, and expression of the retrotransposon *foxy* may provide a mechanism for rapid adaptation, and hence pathogenicity evolution, in Fusarium populations.

6. Detail how your research has addressed the Corporation's three Outputs - Economic, Environmental and Social?

This research has provided a platform for ongoing work into the development of novel strategies for improving the resistance of cotton to Fusarium wilt disease. It therefore, if capitalised on, has the potential to contribute to the ongoing viability of the cotton industry. This would be of economic benefit to the cotton industry.

7. Provide a summary of the project ensuring the following areas are addressed:

a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.)

b) other information developed from research (eg discoveries in methodology, equipment design, etc.)

c) are changes to the Intellectual Property register required?

We have contributed to the development of microarray technology for the investigation of gene expression in cotton. We have generated an extensive range of sequenced cDNA clones found to be involved in cotton metabolism, gene regulation, development and defence. This collection is a valuable resource for other cotton researchers. We have already contributed genes to other research programs, such as the modification of cottonseed oil. In the long term, this work could provide material for the development of breeding strategies for the generation of cotton with improved genetic traits.

No changes to the Intellectual Property register are required.

8. Detail a plan for the activities or other steps that may be taken:

(a) to further develop or to exploit the project technology.

(b) for the future presentation and dissemination of the project outcomes.

(c) for future research.

The observations here should be regarded as indicators of genes likely to play a role in the development of resistance or susceptibility in cotton and pathogenicity in Fusarium. In order to generate a more reliable picture of the gene expression changes associated with different infection outcomes, it would be desirable to investigate gene expression changes in a system where there is greater discrimination between susceptibility and resistance. The wild Australian cottons, particularly *G. sturtianum* could provide this system.

Various approaches could be employed to extend the results obtained in this work. Analysis of gene expression can only demonstrate an association of particular genes with resistance or susceptibility. In order to demonstrate the role of a particular gene in the expression of resistance, it would be necessary to change its expression levels experimentally and ascertain the effect that this change has on resistance. As this would be difficult in cotton, due to the inefficiency of existing transformation protocols, it would be desirable to perform these investigations in a model system, such as Arabidopsis. The CRDC funded a pilot project that achieved the establishment of a model Arabidopsis/Fusarium infection system, but funding to continue this work was not available. The importance of aquaporins could be investigated further by comparing aquaporin gene expression changes under water stress and after infection, and by performing studies looking at the correlation between plant water relations and aquaporin gene expression and aquaporin protein levels.

Investigation the importance of transposons in the generation of variation within Fusarium populations could yield valuable information regarding the development of pathogenicity. Brubaker and coworkers have extensive collections of Fusarium isolates that could be used to track the movement of *foxy* and associate this with pathogenicity. Further work on the investigation of genes involved in pathogenicity and infection processes could be performed utilising the inactivation of the fungal genes identified in this study. Protocols for transformation of Fusarium and for inactivation of specific genes have been established in

this laboratory, demonstrating the feasibility of this approach. In addition, further work with randomly generated Fusarium mutants could be performed to identify additional pathogenicity genes.

9. List the publications arising from the research project and/or a publication plan.

Publications:-

1. Caitriona Dowd, Iain W. Wilson and Helen McFadden. Gene Expression Profile Changes in Cotton Root and Hypocotyl Tissues in Response to Infection with *Fusarium oxysporum* f. sp. *vasinfectum.*, **Molecular Plant Microbe Interactions**, accepted for publication.

2. Helen McFadden, Iain W. Wilson, Robin Chapple and Caitriona Dowd. *Fusarium oxysporum* f. sp. *vasinfectum* genes expressed in infected cotton tissue. For submission to **Molecular Plant Microbe Interactions,** passed for submission by Plant Industry panel.

3. Helen McFadden, Dean Beasley & Curt L. Brubaker. *Gossypium sturtianum* is a potential source of Fusarium wilt resistance in cotton breeding. **Euphytica**, submitted.

4. Caitriona Dowd, Iain W. Wilson and Helen McFadden. Gene Expression Profile Changes in the Stems of a Resistant and a Susceptible Cotton Cultivar in Response to Infection with *Fusarium oxysporum* f. sp. *vasinfectum.* In preparation.

Other:

We made several presentations at Fusarium researcher meetings, at the Australian Cotton Conference, and at the International Congress of Plant Pathology, Christchurch, Feb 2003.

10. 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 or the Australian community.

This work has achieved a valuable first step in the development and application of a technology for the investigation of cotton and Fusarium wilt pathogen gene expression. We have identified several potential areas for further work, such as investigation of the role of genes for aquaporins in wilt symptom development and investigation of the role of retrotransposons in facilitation of the development of pathogenicity in Fusarium. However, further investigation of these observations is required before they could be applied to the development of novel sources of resistance to Fusarium wilt.

Part 4 – Final Report Executive Summary

Provide a one page Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

Fusarium wilt disease has the potential to cause significant yield losses and the removal of some areas from cotton production. It is therefore likely to impact on the long-term sustainability of the cotton industry. Conventional breeding techniques have made some progress towards generating resistance to Fusarium wilt in the cotton plant, but breeding efforts would be enhanced by knowledge of the factors that contribute to resistance. In addition, knowledge of the factors controlling the pathogenicity of the pathogen could provide the basis for the development of novel techniques that allow minimisation of the impact of the disease.

Resistance to the wilt pathogens is complex and probably controlled by many genes. This makes the task of breeding for resistance complex and difficult. In order to determine suitable approaches for improving cotton's resistance by breeding or by the use of genetic engineering, we need an improved understanding of the existing plant responses that are effective at giving some resistance to Fusarium wilt. Identification of the genes deployed by cotton during infection by the Fusarium wilt pathogen, particularly those associated with the response of more resistant species or cultivars, could indicate new targets for effective breeding or for manipulation by genetic engineering. In this project, we aimed to develop tools for the large-scale study of gene expression in cotton and Fusarium, and to apply these tools to investigate gene expression in the host and the pathogen during the infection process.

During the course of this project, we developed effective microarray and model infection systems for the analysis of gene expression in Fusarium-infected cotton. This is a resource of potential use to the cotton industry in the identification of potential targets for generation of improved resistance to Fusarium wilt.

The most striking observation made during analysis of gene expression patterns was that in infected seedlings, gene expression changes in roots and hypocotyls appear to be different.

We found that repression of gene expression, particularly repression of genes for water-regulating proteins, such as aquaporins, was consistently observed in roots and hypocotyls. This repression appears to be associated with susceptibility. This identifies aquaporins as potentially important in the development of wilt symptoms.

In the Fusarium wilt pathogen, expression of a homologue of the *AtsC* gene from Agrobacterium may be important for pathogenicity, and expression of the retrotransposon *foxy* may provide a mechanism for rapid adaptation, and hence pathogenicity evolution, in Fusarium populations.