Australian Government
Cotton Research and
Development Corporation

FINAL REPORT 2008

If you are participating in the presentations this year, please provide a written report and a copy of your final report presentation by 31 October.
If not, please provide a written report by 30 September.

Part 1 - Summary Details

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

CRDC Project Number: ANU9

Project Title: Testing transgenic cotton carrying the I-2 resistance gene for resistance to Fusarium wilt

Project Commencement Date: 1/10/2006 **Project Completion Date:** 30/09/2008
CRDC Program: 3 Crop Protection

Part 2 – Contact Details

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Background

1. *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) has emerged as a very serious pathogen of cotton, which apparently lacks good sources of endogenous resistance to *Fusarium* wilt. In contrast, there are several well defined genes in tomato for resistance to the related pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and tomato has the best characterised system of plant interaction with *Fusarium*. The tomato *I-2* gene for resistance to FOL has been cloned and found to encode a coiled-coil - nucleotide-binding site - leucine-rich repeat (CC-NBS-LRR) protein similar to proteins encoded by a number of resistance genes to various plant pathogens in various plant species.

   Under previous CRDC funding, we engineered the *I-2* gene to allow its expression/over expression under the control of a strong constitutive promoter (the *35S* promoter from cauliflower mosaic virus) and, in collaboration with CSIRO Plant Industry, attempted to produce *35S:I-2* transgenic cotton. However, we were unable to generate transgenic cotton lines containing *35S:I-2*. We interpreted this result to indicate that over-expression and/or inappropriate temporal or spatial expression of the I-2 protein caused inappropriate activation of cotton defence mechanisms leading to cell death. Interestingly, this finding is consistent with the expression of some disease resistance genes in different plant species e.g. the flax *L6* resistance gene expressed in tobacco causes the constitutive activation of tobacco plant defences.

   We continued to test *I-2* function in cotton by *Agrobacterium*-mediated transient expression of the *35S:I-2* gene in cotton leaves. We found that over expression of *I-2* in cotton leaf tissues resulted in necrosis consistent with activation of cotton defences. Again, this finding is consistent with results that would be expected following over-expression of an active resistance gene. More importantly, it indicated that *I-2* was biologically active in cotton.

   Given that over expression of *I-2*, caused inappropriate activation of cotton defence mechanisms, we explored the possibility of using the *I-2* promoter in place of the *35S* promoter. A glucuronidase (*GUS*) reporter gene was fused to the *I-2* promoter and the fusion construct was expressed in hairy roots of cotton induced by *Agrobacterium rhizogenes*. Staining for GUS activity showed that the *I-2* promoter was active and showed a similar pattern of expression in cotton to that in tomato i.e. in cells adjacent to the vascular tissues of the root. We therefore made a DNA construct with the *I-2* gene driven by its own promoter for use in cotton transformation experiments.

   Following the conclusion of our previous CRDC funding, we proceeded, in collaboration with CSIRO Plant Industry, to produce transgenic cotton with the *I-2* gene under the control of its own promoter. Twenty-two T0 lines were produced, with 9 of these identified as independent lines. In this project, we have analysed the progeny of these primary transformants to determine how many transgene loci are present. Many of the primary transformants carry multiple *I-2* loci. All *I-2* lines were screened for their resistance/tolerance towards FOV in glasshouse trails. Whilst the preliminary results from an initial glasshouse trial were encouraging, later FOV bioassays demonstrated that the possession of the *I-2* gene appeared to have no effect on resistance/tolerance in cotton against FOV.

Objectives

2\.

***Project Objective 1**: Produce I-2 transgenic cotton lines for glasshouse trials*

Twenty-two T0 lines were produced and nine of these were identified as independent lines. Homozygous single *I-2* locus lines were produced for each of these nine lines and analysed for FOV disease resistance/tolerance in glasshouse trials. Thirteen other segregating and multilocus lines were also analysed.

***Project Objective 2**: Characterisation of cotton lines for expression of I-2 gene*

Where RNA yields permitted, RT-PCR was used to analyse the presence or absence of *I-2* mRNA in the root tissues of two seedlings from each of the 22 lines. A housekeeping gene *histone3* was used for comparison. Expression of *I-2* was confirmed in most of the root tissues sampled from *I-2* transgenic plants. *I-2* expression was not detected in seedlings from null sibling lines, and some seedlings from segregating lines. Expression of *I-2* in the cotyledons and hypocotyls of several *I-2* cotton seedlings was also confirmed.

***Project Objective 3**: Characterisation of I-2 cotton lines for defence response gene expression*

We used qPCR analysis of the expression of three defense response genes (DRGs): PR5, PR10, and endochitinase precursor (EC) in hypocotyl tissue. Quantitation was performed against the polyubiquitin (UB) gene as the housekeeping gene. We could not find any evidence for changes in DRG expression in seedlings from the nine independent homozygous lines. Levels of PR5 and EC remained low, and at the lower limits of detection in most of the seedlings analysed.

***Project Objective 4** Analysis of I-2 cotton resistance to FOV in glasshouse trials*

A small FOV trial was conducted early in the 2006/2007 growing season. Unfortunately storm damage to CSIRO facilities prevented further trials in that season. A full analysis was completed in the 2007/2008 season. All 22 lines, including both single and multi-locus lines, were analysed.

Methods

3\. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

Production of I-2 transgenic cotton lines for glasshouse trials

I-2 cotton lines were generated previously in collaboration with CSIRO Plant Industry. The *I-2* gene used included the native *I-2* promoter, which we have shown previously to be functional in driving GUS expression in cotton roots. Primary transformants (T0) were isolated from tissue culture and selfed to produce the T1 generation. Seedlings (T1) were screened for the presence of the *I-2* gene using PCR amplification of a small region of the 5' end of the *I-2* coding sequence. Analysis of both the segregation pattern in the seedlings, and transgene counting via qPCR analysis, was used to distinguish between single and multilocus lines. Homozygous lines were identified by similar analysis of T2 seedlings using PCR.

Transgenic lines for further analysis were identified that had both a T1 population that inherited the I-2 gene in a ratio consistent with a single locus insertion, and a qPCR transgene count in the genomic DNA of the T0 parent of 1. These were used initially as the focus for further RT-PCR and FOV bioassays.

qPCR analysis of transgene number

Due to limits in time and resources we chose to use qPCR to analyse the number of transgenes present within the genome of individual *I-2* cotton lines. This method is potentially far faster than Southern blotting for transgene analysis, and has been used before for similar purposes in other systems (for example see: Chen, Q., et al, 2006 Screening of copy number polymorphisms in human h-defensin genes using modified real-time quantitative PCR, *J. of Immunol. Meth.*, 308:231– 240.).

PCR primers and TaqMan probes towards the 3' coding region of the *I-2* gene were designed for use in multiplex qPCR analyses. The housekeeping gene chosen as a copy-number control was stearoyl-ACP desaturase (SAD1), which has already been used for qPCR work, and which has published primer and probe sequences available. As the comparator, we chose DNA from a transgenic *I-2* line which had been previously characterised as containing a single-locus *I-2* insertion.

RT-PCR analysis of cotton lines for expression of I-2 gene

We chose RT-PCR analysis to determine the pattern of *I-2* gene expression in the roots of T1 seedlings. This offered the possibility of higher throughput than Northern blot analysis. Total RNA was extracted from the roots of 2-3 week old seedlings, reverse translated to cDNA, and used in PCRs. PCR primers were used that spanned the 5' UTR (275 bp amplicon) of the *I-2* gene. For comparison the housekeeping gene *histone3* was used as an internal control, with PCR primers that spanned an intron (412 bp amplicon).

Consistent and repeatable amplification of the *I-2* RT-PCR product could only be achieved with 35 PCR cycles, whereas amplification of the housekeeping gene was achieved at 25 cycles. For this reason it was difficult to get reliable semi-quantitative measures of *I-2* gene expression. Therefore we used the RT-PCR results as an indication of whether the I-2 gene was expressed (generation of a specific 275 bp *I-2* amplicon) or not expressed (absence of *I-2* amplicon after 35 cycles).

We analysed two seedlings from each of the chosen transgenic lines to determine whether-or-not the line was expressing *I-2*. In a number of cases we could only detect *I-2* expression in one of the two seedlings. Such results were interpreted as indicating that expression of *I-2* in the transgenic line was occurring at a very low level. Seedlings from a null line were used to confirm that the RT-PCR was specific for *I-2*.

qPCR analysis of expression of defence response genes (DRGs)

Nine cotton lines expressing *I-2* were chosen for analysis of defence response gene expression. We identified genes for PR10, PR5b, and endochitinase precursor (EC) as potentially useful for measuring defence response in cotton lines. Primers and probes were designed for qPCR analysis and qPCR experiments were undertaken to measure the extent of up- or down-regulation of these transcripts in transgenic seedlings.

Again we used multiplex Taqman qPCR assays to determine the amounts of DRG expression, and used the housekeeping gene for polyubiquitin (UB) as the internal control. We designed both primers and probes for PR10, PR5b, EC and UB targets, and used these together in 4-plex qPCR assays. Data collected from cDNA taken from null seedlings was used as a comparator, so that the results were expressed as a ratio compared to the non-transgenic siblings.

Analysis of I-2 cotton resistance to FOV in glasshouse trials

FOV bioassays were conducted by Dr Augusto Becerra Lopez-Lavalle of CSIRO Plant Industry, using methods developed at CSIRO-PI. The main trial (2007/08) involved the assay of all 22 *I-2* cotton lines, 22 sibling null lines, and 4 cotton varieties of differing susceptibility to FOV (including Siokra 1-4, Coker, Sicot 189, and Sicot F-1). Rankings were assigned to each test group according to the severity of symptoms that developed during exposure to FOV. Final results were given as a rank score, such that comparisons between the transgenic lines, null lines, and the control varieties could be made.

Results

4.

Production of I-2 transgenic cotton lines for glasshouse trials

Breeding of the *I-2* cotton lines to be analysed in this study has been completed. Twenty-two lines, including nine homozygous single-locus lines, were produced. Several other T0 individuals failed to produce viable seed, and could not be analysed. Homozygous lines were identified by qPCR analysis of seedlings to first identify T1 lines that segregated for a single locus and then to identify homozygous T2 seedlings. Analysis of transgene number was conducted using qPCR analysis of *I-2* number against the *SAD1* gene, which is present as one copy in each of the four haploid cotton genomes.

The qPCR analysis was performed using the ΔΔCt method over a DNA concentration range where the amplification efficiencies of the different PCRs were nearly identical. Analysis was performed using three-or-more technical replicates for each sample of T0 DNA.

Characterisation of cotton lines for expression of I-2 gene

RT-PCR analysis of I-2 gene expression in the roots of two seedlings from all but one of the 22 transgenic cotton lines being used in glasshouse trials was carried out. The data suggested that all but one of the 21 lines showed evidence for *I-2* expression, although in most cases *I-2* expression was low, and often difficult to detect. This was expected because disease resistance genes are often expressed at low levels under their own promoters. Time constraints prevented us from performing analyses on more than 2 plants from each line.

Characterisation of I-2 cotton lines for defence response gene expression

Analysis of PR10, PR5b, and endochitinase precursor (EC) gene expression in the hypocotyl tissues of seedlings from independent single-locus lines was undertaken using qPCR analysis. Hypocotyl tissues have been used in studies of cotton defence gene expression in response to FOV infection. Yields of RNA from cotton hypocotyl were often low in our hands. Spectroscopic analysis of purified RNA showed a high level of purity, and the cDNA synthesised yielded RT-PCR amplicons of the expected size under standard PCR conditions (35 cycles). qPCR analysis demonstrated that the housekeeping gene (HKG), polyubiquitin (UB) started amplification at around 20-25 cycles in cDNA from hypocotyl, whereas amplification of the genes of interest (PR10, PR5b, and EC) often started at 30 to 35 cycles, indicating that they were at much lower abundance. Of the three genes of interest, PR10 gave the most consistent amplification efficiencies, and earliest amplification. The order of amplification start was: UB<PR10<PR5b<EC (i.e. UB PCR products were detected at a lower number of PCR cycles than any of the other genes).

These initial findings indicate that the levels of PR5b and EC expression in hypocotyl tissues were low, and remained so regardless of whether the seedlings were transgenic (*I-2*) or sibling null lines. Whilst PR10 values were more reliable (consistent amplification efficiency), analysis of the expression data from one seedling from each of eight single-locus independent lines demonstrated that the expression levels were similar in both the *I-2* cotton line and its sibling null comparator.

We could not, therefore, find any evidence that PR10, PR5b or EC levels are increased in the transgenic *I-2* lines compared to the non-transgenic null lines. An improved methodology resulting in a higher RNA yield and better performance for the analysis of EC expression might yield more reliable data. However, it is also likely that the target genes chosen are expressed at only very low levels in *I-2* cotton hypocotyls.

Analysis of I-2 cotton resistance to FOV in glasshouse trials

Early observations of seedlings in the 2006/07 FOV bioassays seemed to imply that multi-locus lines may possess some tolerance to FOV infection. However analysis of seedling tolerance at the end of this bioassay run yielded results that did not live up to the early expectations. In an effort to understand whether single- or multi-locus *I-2* lines differed in their tolerance to FOV, we included all available *I-2* cotton lines in our 2007/08 FOV bioassays. Cotton lines with varying tolerance to FOV: Siokra 1-4, Coker, Sicot 189, and Sicot F-1 were also included.

Most of the lines studied, including the transgenic lines and their sibling nulls, were more susceptible to FOV than the Coker parent. One I-2 cotton line (366-12:1210) was the most susceptible line overall, being more susceptible than Siokra 1-4, whereas the most tolerant of the I-2 cotton lines, 366-22:373, had a score (4.2) in between that of Coker (3.5) and Sicot 189 (4.7). Forty one lines tested (including transgenic and sibling nulls) were more susceptible than Coker, whereas only 3 lines were intermediate in susceptibility between Coker and Sikot 189.

To determine whether the presence of the *I-2* gene was responsible for any observable change in FOV tolerance, the scores for the sibling null lines were subtracted from that of the corresponding transgenic lines. When this was done, there was no detectable influence of the *I-2* gene on tolerance to FOV in cotton.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

One of the main outcomes of this study was to determine the suitability of *I-2* cotton as a source of FOV resistance for breeding purposes. From the results of our study, particularly those from the glasshouse assays, the *I-2* gene in its present form appears to be unsuitable for use in combating FOV infection in seedlings. The fact that *I-2* appears to have some activity in cotton, is being expressed in the roots and hypocotyls of seedlings, and yet appears to have no detectable influence on tolerance to FOV will provide useful knowledge to future efforts to engineer cotton resistance to FOV. Since there are potentially a number of different avenues available to engineer resistance into cotton, understanding which pathways do not yield resistance provides better opportunities for future attempts to introduce disease resistance genes successfully into cotton.

6. Please describe any:-
   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.); and
   c) required changes to the Intellectual Property register.

None

Conclusion

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

Although introduction of *I-2* into cotton appears to have had little effect on resistance/tolerance to FOV, a lot is still not known about the processes that lead to FOV resistance/tolerance in cotton. For instance, *I-2* in tomato is likely to function in association with systemic acquired resistance (SAR), which requires the accumulation of salicylic acid (SA). However, it is not known how important SAR is to FOV resistance in cotton. It is possible that resistance in cotton may require induced systemic resistance (ISR), which requires a jasmonic acid (JA)-dependent pathway for establishment. Ethylene (ET) is a complicating factor, which appears to have a role in defence gene induction but a significant negative role in resistance to FOV in cotton. Hence, a better understanding of the contributions of each of the JA, ET, and SA-inducible systems on FOV resistance may be required before the genetic modifications necessary to produce FOV tolerance can be predicted.

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:
   (a) to further develop or to exploit the project technology. **None.**
   (b) for the future presentation and dissemination of the project outcomes. **See 8A below.**
   (c) for future research.

Future studies could perhaps be directed towards understanding the roles or influences of SA, JA and ET on FOV tolerance in cotton. At the same time, microarray analyses of genes up- and down-regulated by SA, JA and ET and in tolerant versus susceptible cotton cultivars could be conducted to see how the various patterns of SA, JA and ET-regulated gene expression relate to tolerance to FOV in cotton. The recent cloning of the FOL *Avr2* gene also offers the possibility of testing the *I-2/Avr2* interaction in cotton. If the interaction triggered the activation of a plant defence response then a number of new research possibilities would be opened up including the possible analysis of defence gene induction in a resistant rather than tolerant reponse to FOV.

9. A. List the publications arising from the research project and/or a publication plan.
   (NB: Where possible, please provide a copy of any publication/s)

We plan to prepare and submit a manuscript describing this work within the next year.

B. Have you developed any online resources and what is the website address?

No

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 oxysporum f. sp. *vasinfectum* (FOV) is a very serious pathogen of cotton, against which cotton has few sources of endogenous resistance. In contrast, there are several well defined genes in tomato for resistance to the related pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Transient over-expression of *I-2* in cotton tissues provided evidence for the biological activity of the *I-2* protein in cotton. This observation, along with experiments that demonstrated that the *I-2* promoter functions in cotton roots, led us to attempt to determine whether *I-2* transgenic cotton would be a suitable source of resistance to FOV. So, in collaboration with CSIRO Plant Industry, we produced transgenic cotton with the *I-2* gene under the control of its own promoter. Twenty-two transgenic lines were produced and variously characterised for locus and transgene number, for *I-2* gene expression, and for activation of defense response genes. All of the *I-2* lines were screened for their tolerance towards FOV in glasshouse trails.

Whilst the early results from an initial glasshouse trial were encouraging, later FOV bioassays demonstrated that the possession of the *I-2* gene appeared to have no effect on tolerance towards FOV. Although this was not the outcome desired, the results suggest that the *I-2* gene, and perhaps other resistance genes that function in association with salicylate-accumulation and systemic acquired resistance, may not be good choices for engineering FOV tolerance. An alternative engineering strategy may be required, perhaps one that makes use of other resistance pathways.