H

January, August & Final Reports

REPORTS

Part 1 - Summary Details

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CRDC Project Number: **CSP104C**

January Report: ☐ Due 29-Jan-01
August Report: ☐ Due 03-Aug-01
Final Report: ☐ Due within 3 months of project completion

Project Title: Evaluation of disease tolerance of transgenic cotton lines containing genes for putative antifungal proteins

Project Commencement Date: 7/99 **Project Completion Date:** 6/01
Research Program: Diseases Weeds

Part 2 – Contact Details

Administrator: Ms Danielle Stiller
Organisation: CSIRO Plant Industry
Postal Address: GPO Box 1600, Canberra, ACT 2601
Ph: 02-6246 5041 **Fx:** 02-6246 5000 **E-mail:** D.Stiller@pi.csiro.au

Principal Researcher: Dr Helen McFadden
Organisation: CSIRO Plant Industry
Postal Address: GPO Box 1600, Canberra, ACT 2601
Ph: 02-6246 5377 **Fx:** 02-6246 5000 **E-mail:** H.McFadden@pi.csiro.au

Supervisor: Dr Jeff Ellis/Dr Tony Pryor (previously Dr Liz Dennis)
Organisation: CSIRO Plant Industry
Postal Address: GPO Box 1600, Canberra, ACT 2601
Ph: 02-6246 5041 **Fx:** 02-6246 5000 **E-mail:** Jeff.Ellis@pi.csiro.au

Researcher 2 Dr Danny Llewellyn
Organisation: CSIRO Plant Industry
Postal Address: GPO Box 1600, Canberra, ACT 2601
Ph: 02-6246 5470 **Fx:** 02-6246 5000 **E-mail:** Danny.Llewellyn@pi.csiro.au

Signature of Research Provider Representative: Blundell

Part 3 – Final Report Format

Project background

Fungal diseases of cotton, in particular *Fusarium oxysporum* f. sp. *vasinfectum,* are important in limiting the yield and production of cotton in Australia. Improvement of crop resistance is a major strategy in the control of the impacts of disease on cotton production. We have therefore conducted a long-term program to incorporate transgenes for putative antifungal proteins into cotton germplasm.

The aim of the project is to improve cotton's tolerance to fungal pathogens. We have targeted the vascular, root-invading pathogens, *Verticillium* and *Fusarium*, but one of the advantages of antifungal proteins is that they may be effective against a range of pathogens. Therefore the material we have generated has the potential to have improved tolerance to several fungal diseases. Our original focus was on *Verticillium* wilt, and our testing of transgenic lines was conducted using this pathogen. However, with the increasing importance of *Fusarium* wilt to the industry, we decided to shift to using *Fusarium* as our test organism. We therefore spent some time putting a *Fusarium*-testing facility and protocol in place.

Project objectives

The objectives listed in the original grant proposal are given below. As indicated, there have been some modifications to these objectives as the work has progressed.

Year 1

1. Generate sufficient seed of double homozygous lines expressing chitinase plus osmotin for glasshouse trials, conduct trials against *Verticillium* wilt and compare with parent lines.
2. Repeat glasshouse trials with osmotin transgenic lines to finalise conclusions about the effectiveness of this line against *Verticillium* wilt.
3. Generate T^0 cotton lines transformed with

- glucanase,
- glucanase plus chitinase,
- glucanase plus chitinase plus osmotin,
- aminocyclopropane deaminase (for reduced ethylene production).

4. Cross glucanase-expressing plants generated above with existing chitinase lines.
5. Test chitinase/V2 Ingard progeny against *Verticillium* in the glasshouse.
6. Select homozygous lines expressing *beta*-purothionin genes and generate seed for glasshouse testing.

Year 2

7. Characterise transgene expression in lines generated in (3) above. Generate homozygous lines and commence glasshouse testing with *Verticillium* and *Alternaria.*
8. Perform glasshouse testing of radish antifungal protein and *beta*-purothionin-expressing lines (generated previously in other projects) against *Verticillium* and *Alternaria.*

Achievements against objectives

Generation of transgenic cotton and testing of lines for resistance to pathogens

(for details of transgenic lines please see Table 1)

Osmotin and osmotin plus chitinase (Objectives 1, 2 and 7)

Homozygous seed of the best osmotin-expressing transgenic line (generated previously) and seed of a line homozygous for both the best chitinase-expressing transgene and the best osmotin-expressing trangene was generated for glasshouse testing. In late 1999 a glasshouse trial to test the double homozygous line expressing both chitinase and osmotin and the osmotin expressing line was conducted. Some reduction in *Verticillium*-induced stunting was observed but the level of stunting obtained was not sufficiently marked for any protective effect to be statistically significant. The test was repeated in March/April/May 2000 using two chitinase lines, the osmotin and osmotin x chitinase lines. Again, the degree of stunting in the non-transgenic control line obtained was insufficient to allow definite conclusions about the effectiveness of the transgenes, but no dramatic reduction in stunting was observed. A further glasshouse test was performed with two osmotin-expressing lines in late 2000 and again no significant protection was observed.

Thus, conclusive results regarding the antifungal activity of the putative antifungal genes were not obtained. However, in the experiments performed, there was no evidence of dramatic improvement in the tolerance of the transgenic lines to *Verticillium* wilt. Therefore we have concluded that the osmotin and chitinase with osmotin lines are probably no better than the chitinase line tested previously that did not show improved fungal tolerance against *Verticillium* or *Fusarium* in the field, although it had shown reduction in disease severity in glasshouse tests.

As reported in July 2000, it was decided not to test the transgenic plants for resistance to *Alternaria*. This was because of difficulties experienced in setting up a glasshouse assay for *Alternaria*, and because of its reduced importance in the cotton industry. We also indicated in the July 2000 progress report that, given the rapid growth in the importance of *Fusarium* wilt to the cotton industry, we should shift our emphasis for testing from *Verticillium* to *Fusarium* and set up a *Fusarium*-testing facility and protocol. This has now been achieved and any further testing of transgenic cotton lines will be against *Fusarium* wilt and not *Verticillium* wilt.

Glucanase, Glucanase plus chitinase, and Glucanase plus chitinase plus osmotin (three-gene construct) (Objective 3).

Despite several attempts over the years, we have been unable to obtain fertile transgenic cotton plants where we could confirm glucanase expression. In the latest batch of transgenic plants we obtained six independently transformed T^0 plants transformed with the glucanase plus chitinase construct. Of these, three showed expression of the glucanase gene using Northern analysis for mRNA transcript levels. All three of these plants were sterile. It has been shown in tobacco that glucanase expression results in male sterility (Hird *et al.*, 2000, **Transgenic Research**, vol 9, p91-102). It is therefore possible that glucanase over-expression is inducing male sterility in cotton, so that we are unable to generate fertile, glucanase-expressing transgenic plants. One of the glucanase plus chitinase T^0 plants showed strong chitinase mRNA expression and barely detectable glucanase mRNA expression and was fertile. Seed from this plant has been sown. Expression of the selectable marker gene (NPTII) has been confirmed and tissue samples for mRNA analysis taken. If expression of

chitinase and glucanase genes is confirmed in these plants, their seed will be tested to select lines to be used for the assessment of their resistance to *Fusarium* wilt.

We obtained 7 independently transformed plants transformed with the 3-gene construct. Of these, 6 showed expression of the selectable marker gene (NPTII). No strong expression of the glucanase gene was detected, 2 showed chitinase gene expression and 4 showed osmotin gene expression using Northern blot analysis. Only one plant showed both chitinase and osmotin expression. Seed from this plant has been sown for further assessment as for the above constructs.

Aminocyclopropane (ACC) deaminase (Objective 3).

Aminocyclopropane (ACC) deaminase enzyme disrupts synthesis of the signal molecule ethylene, which may be responsible for wilting and leaf drop symptoms. It was thought that reduction in ethylene production might therefore ameliorate wilt symptoms. However, as reported in February 2000, work on this objective was discontinued. As indicated, the sequence of the construct (supplied by a collaborator in South Africa) was found to contain errors. It was not considered worthwhile for us to remake the construct ourselves.

Chitinase x glucanase (Objective 4).

This cross could not be performed because confirmed glucanase-expressing plants were not obtained. However, we have one line that may be expressing chitinase and glucanase, as indicated above.

Chitinase x V2 Ingard (Objective 5).

As reported in February 2000, seed is available but testing for *Verticillium* resistance was not performed because of anecdotal evidence of increased susceptibility to *Fusarium* wilt associated with Ingard cultivars. The objective was discontinued.

Beta-purothionin (Objectives 6 and 8)

Transgenic lines transformed for *beta*-purothionin expression (generated previously) were selected for selectable marker gene expression (Basta resistance). Basta-tolerant lines were assessed for *beta*-purothionin gene expression using Northern analysis. A homozygous, highly expressing line was selected and tested for improved resistance to *Verticillium* wilt. No protection against disease severity was observed.

Radish antifungal protein (Objective 8)

Only one line transformed with radish antifungal protein (Rsafp) was available (from previous work). Our objective was to characterise this line and, if appropriate, test for resistance to pathogens. Progeny of the line were sown, assessed for selectable marker gene expression (NPTII) and then for transgene expression using Northern analysis. Despite readily detectable expression of the selectable marker gene, no expression of the Rsafp gene was detected, therefore no pathogen testing was undertaken.

Additional topics added during the course of the project

Sulphur as a phytoalexin in resistant *G. hirsutum* infected with *Verticillium.*

Sulphur has only recently been identified as a potential source of resistance in plants infected with vascular wilt diseases. Establishing its importance in cotton may provide new opportunities for developing selection methods for resistant germplasm and improve our understanding of which processes actually contribute to wilt resistance. In collaboration with

Dr Richard Cooper at Bath University, we performed an experiment to determine the role of sulphur production in cotton's defence responses to *Verticillium* infection. We compared the levels of sulphur in a resistant (Sicala V2) and a susceptible (CS50) cultivar after *Verticillium* inoculation and water inoculation (control). Increased levels of sulphur were identified in *Verticillium* infected cotton stems, with greater increases in the resistant cultivar.

Expression of NPR1 gene in transgenic cotton

This gene has been implicated as being an important switch in the activation of defence responses in plants. In other plant systems, when the NPR1 gene is over-expressed, plants are primed for defence. In other words, defence responses are activated more rapidly than in control plants. There is a potential to combine the effects of this gene with treatment with chemical activators of defence, such as bion (BTH). We obtained two independently transformed T^o plants. Northern analysis demonstrated NPR1 gene expression. One of the plants was sterile, but seed from the other has been planted for further analysis.

RT-PCR analysis of defence gene expression in *Verticillium*-infected cotton

In order to develop our understanding of which aspects of plant responses to *Verticillium* wilt are important for defence, we have compared the increases in expression levels of several pathogenesis-related genes in a resistant cultivar (Sicala V2) and a susceptible cultivar (CS50) after *Verticillium* infection using RT-PCR. We found that although mRNA for cadinene synthase, basic chitinase, and PR10 genes was induced after pathogen inoculation, there were no detectable differences in the timing or extent of this induction. Thus we conclude that it is unlikely that the increased levels of expression of these genes is responsible for the observed resistance of Sicala V2 compared with CS50 to *Verticillium* infection.

Outcomes of the project

The main outcomes of the project are:-

- a collection of seed for transgene-expressing lines of cotton as outlined in Table 2.
- the observation that gene expression levels for several classical pathogen-response genes increase in response to Verticillium inoculation in cotton stems, but that this induction does not appear to account for the observed differences in susceptibility of the cultivars tested (published).
- an established Fusarium-testing glasshouse facility to test for Fusarium resistance.

Research Project impact on the Corporations three outputs: Sustainability, profitability and international competitiveness, and/or people and community

If this project had generated cotton with improved resistance to vascular wilt diseases, it would have made a valuable contribution to reduction of the deleterious effects of disease on cotton production. Such an outcome had the potential to contribute significantly to the profitability and economic sustainability of the cotton industry. It is still possible that some of these transgenic lines may be identified in ongoing testing as having some improved resistance to *Fusarium* wilt.

Project methodology

Cotton transformations were carried out by Robin Chapple and by Danny Llewellyn's transformation team using established protocols. Assessment of putative transformants for the

expression of the selectable marker gene for NPTII was based on a standard assay for phosphotransferase enzyme activity. Basta-resistant plants were selected by spraying with herbicide. Assessments of gene expression were made using Northern blot analysis for mRNA transcript for levels of gene expression. Chitinase assays, and early glucanase assays were based on analysis of resulting enzyme activity using published procedures. Assessment of resistance to *Verticillium* wilt was performed using pot tests in the glasshouse, with the main assessment of disease severity being based on the degree of stunting observed. The assessment of resistance to *Fusarium* wilt is performed using a glasshouse pot test based on procedures developed by Bo Wang (UQ) and Natalie Moore (QDPI). Assessment is based on foliar symptoms, stunting and vascular browning.

Discussion of results

As outlined in detail above, we have generated a range of transgenic cotton plants expressing genes for various putative antifungal proteins. As anticipated, not all transformations proceeded rapidly enough for us to have generated sufficient, characterised material for all gene constructs to assess their performance against vascular wilt disease. (Completion of this assessment was not included as a specific project objective). For those transgenic lines that were tested, no significant degree of protection against *Verticillium* wilt was observed. This is due, at least in part, to problems with obtaining a sufficient level of *Verticillium* wilt symptoms in the glasshouse assay. Thus we have discontinued testing with *Verticillium* and have developed a facility for testing for *Fusarium* resistance. It is planned that transgenic cotton lines will be tested for resistance to *Fusarium* wilt in the future.

Impact of conclusions on the cotton industry

As this project has not identified any genes for putative antifungal proteins that have a dramatic effect against *Verticillium*, it is not likely that this project will have a great impact on the cotton industry at this time. There is, however, a chance that some of these lines will be found to have an effect against *Fusarium* when tested in the glasshouse against this pathogen.

Publications arising from the research project

The first two articles were submitted in 1997, but appeared in print in 2000.

2000 **McFadden, H.**, de Feyter, R., Murray, F., Grover, A., Llewellyn, D., Dennis, E., and Peacock, W.J. Genetic Engineering Approaches to the Improvement of Cotton's Tolerance to *Verticillium* Wilt.
In: Advances in *Verticillium* Research and Disease Management, pp 187-191. Tjamos, E.C., Rowe, R.C., Heale, J.B. and Fravel, D.R. (Eds.) The American Phytopathological Society, St. Paul.

2000 **McFadden, H.** Prospects for Controlling Vascular Wilt Diseases of Cotton and other Crops by Genetic Engineering.
In: Advances in *Verticillium* Research and Disease Management, pp 166-172. Tjamos, E.C., Rowe, R.C., Heale, J.B. and Fravel, D.R. (Eds.) The American Phytopathological Society, St. Paul.

2000 **McFadden H.**, de Feyter R. and Llewellyn, D. Molecular biology approaches to understanding and controlling *Fusarium* wilt in cotton.
In: Proceedings of the 10th Australian Cotton Conference (Brisbane, Queensland: August 16-18, 2000)

2000 **McFadden, H.G.**, Lawrence, G., and Dennis, E.S. Changes in chitinase activity in flax, *Linum usitatissimum*, in response to inoculation with virulent or avirulent strains of flax rust, *Melampsora lini*. Australasian Plant Pathology 30, 27-30.

2001 **McFadden, H.G.**, Chapple, R.M., de Feyter, R. and Dennis E.S. Expression of pathogenesis-related genes in cotton stems in response to infection by *Verticillium dahliae*. Physiological and Molecular Plant Pathology 58, 119-131.

2001 Cooper, R.M., Williams, J.S. Hall, S.A., Hawkesford, M.J., **McFadden, H.G.** and Beale, M.H. Elemental sulfur is produced by resistant genotypes of several hose species in response to *Verticillium* and other vascular pathogens and may be xylem-specific. Poster to be presented at the International *Verticillium* Conference, Cordoba, Spain, November 2001.

Intellectual Property register

No change required.

Table 2
Homozygous transgene-expressing cotton lines

chitinase	The two best independent chitinase-expressing lines, single gene insertion (Southern blot and segregation), strong expression confirmed by enzyme assay in roots and leaves and RNA analysis (Northern blot)	One line field tested, both glasshouse tested against *Verticillium*
osmotin	The two best independent osmotin-expressing lines determined by RNA expression (Northern blot)	glasshouse tested against *Verticillium*
chitinase x osmotin	A line from chitinase x osmotin found to be homozygous for both genes, shown by PCR and chitinase assay. Presence of both genes confirmed by Southern blot (DNA)	glasshouse tested against *Verticillium*
thionin	The best thionin-expressing line, expression detected by Northern blot	glasshouse tested against *Verticillium*

Other transgene-expressing lines (not homozygous)

truncated glucanase plus truncated chitinase	Weak glucanase expression and strong chitinase expression detected by Northern blot in T^0 plants	T^1 seed harvested and T^1 plants sown
chitinase plus osmotin	The only line from tranformation with the 3-gene construct to yield multiple gene expression. Expression detected by Northern blot.	T^1 seed harvested and T^1 plants sown
NPR1	Strong NPR1 expression detected by Northern blot in T^0 and T^1 plants	T^1 seed harvested and T^1 plants sown

Part 4 – Final Report Plain English Summary

Fungal diseases of cotton, in particular *Fusarium oxysporum* f. sp. *vasinfectum,* are important in limiting the yield and production of cotton in Australia. Improvement of crop resistance is a major strategy in the control of the impacts of disease on cotton production. The aim of the project is to improve cotton's tolerance to fungal pathogens. We have therefore continued with a long-term program to incorporate genes for putative antifungal proteins into cotton germplasm. The aim of this project was to finish off, as much as practical, the generation of transgenic plant lines transformed with genes for putative antifungal proteins, and to test those lines already generated in our work, and by others in the division.

We have targeted the vascular, root-invading pathogens, *Verticillium* and *Fusarium*, but one of the advantages of antifungal proteins is that they may be effective against a range of pathogens. Therefore the material we have generated has the potential to have improved tolerance to several fungal diseases. Our original focus was on *Verticillium* wilt, and our testing of transgenic lines was conducted using this pathogen. However, with the increasing importance of *Fusarium* wilt to the industry, we decided during the course of the project to shift to using *Fusarium* as our test organism. We therefore spent some time putting a *Fusarium*-testing facility and protocol in place.

The testing that was performed against *Verticillium* failed to identify any transgenic lines with particularly promising reduction in the severity of *Verticillium* wilt. This was compounded by difficulties in obtaining good levels of *Verticillium* disease (a problem rarely encountered with the more aggressive *Fusarium* pathogen). It is still possible that some of the transgenic lines generated may be identified in ongoing testing as having some improved resistance to *Fusarium* wilt. We still have several lines that have not yet progressed far enough for testing that may turn out to be of interest, in particular the lines containing genes for more than one anti-fungal protein, and the line transformed with the gene for the potential "switch" protein, NPR1.

In other work associated with the project, we investigated the expression levels of several pathogenesis-related genes in a resistant and a susceptible cultivar after *Verticillium* infection. We found that although mRNA levels for cadinene synthase, basic chitinase, and PR10 genes increased after pathogen inoculation, there were no detectable differences in the timing or extent of this increase. We concluded that these genes are not the primary genes responsible for the observed resistance to *Verticillium* infection. This result is consistent with the disappointing performance of antifungal proteins in transgenic cotton observed so far. In a collaborative project, elemental sulphur was found to be associated with the response of the more resistant cultivar. This suggests a new avenue that might be explored in the search for improving cotton's defence responses. Ongoing work in a new project using microarray technology is progressing well and may identify other potential targets for genetic manipulation.

The main outcomes of the project are:-

- a collection of seed for transgene-expressing lines of cotton as outlined in Table 2.
- the observation that gene expression levels for several classical pathogen-response genes increase, in response to Verticillium inoculation in cotton stems, but that this induction does not appear to account for the observed differences in susceptibility of the cultivars tested (published).
- an established Fusarium-testing glasshouse facility to test for Fusarium resistance.

Table 1: Summary of progress of recent transformation experiments in cotton

Gene	Construct	Transformation	T0's	NPTII	Chitinase	Glucanase	Osmotin	T1 seed
Trunc glucanase plus chitinase	Rob 11678	4 Robin	4.16	++		T0 enzyme+ve		yes/gluc -ve
			4.47					yes
		12 Robin	None					
		T10 Pinghua	None					
Glucanase	Rob 11121	3 Robin	3.37.1	?		T0 enzyme+ve		yes/gluc -ve
			3.37.2	?		T0 enzyme+ve		yes/gluc -ve
		6 Robin	6.2					sterile
Trunc glucanse plus trunc chitinase	Rob 11733	5 Robin	None					
		14 Robin	14.2.1	++	RNA ++	RNA weak		T1 seed sown, sampled for RNA
			14.2.2	+	RNA ?	RNA -ve		yes
			14.3					yes
			14.3.1	+	RNA ++	RNA -ve		yes
			14.3.2	++	RNA +	RNA +		sterile
			14.3.3	++	RNA +	RNA +		sterile
			14.3.4	++	RNA +	RNA +		sterile
Three-gene construct	Rob	289 Pinghua	in progress					
		13 Robin	13.29	-	RNA -ve	RNA -ve	RNA +ve	yes
			13.12.1	+	RNA -ve	RNA -ve	RNA -ve	yes
			13.12.2	+	RNA ?	RNA -ve	RNA -ve	yes
			13.20	+	RNA +ve	RNA ?	RNA +ve	T1 seed sown, sampled for RNA
				++	RNA +ve	RNA -ve	RNA ?	yes
			13.35.2	++	RNA ?	RNA -ve	RNA +ve	sterile
			13.35.1	++	RNA ?	RNA ?	RNA +ve	yes
					NPR1			
NPR1	Ian Dry	9 Robin	9.28	+	RNA ++ve			sterile
			9.46		RNA -ve			
		15 Robin	15.3	?	RNA +ve			T1 seed sown -plants RNA +ve
		330 Erika/ Lisette	some plants in SH pots and soil					