Report Cover Sheet for Annual & Final Reports

The following Reporting Requirements MUST BE MET

All Projects
You must submit an **ANNUAL PROGRESS REPORT** by the first Friday in February 1999, detailing the progress of your research. NOTE: IF you are seeking continuation of funding for 2000–2001 for the project, this report will form the basis for CRDC's consideration of ongoing funding. Please complete the budgetary requirements if this is a continuing project.

Terminating Projects
A **FINAL REPORT** must be submitted within three months of completion of the project. This applies in **ALL** cases including research projects, travel, conference attendances, postgraduate, postdoctoral and funded capital items.

Tick Report Purpose

Annual Progress Report *(Due 1st Fri Feb. to determine continuation of funding)*

Final Report (Due 30 September or 3 months after completion of project)

Final	

Actual start date: ***July 1997***

Anticipated completion date: ***June 1999***

OFFICE USE ONLY:
Date of receipt:

Project title (as per original application)

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

CRDC Project Code **CSP86C**

CRDC Responsible Director (if known)

Organisation CSIRO

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Final Report

What was the background of the project?

Plants respond to infection by increasing the levels of several proteins, known as PR (pathogen response) proteins (Ward *et al.*, 1991). Some of these proteins have been shown *in vitro* and *in vivo* in transgenic plants (eg Broglie *et al.*, 1991) to have antifungal activity. Synergism between different antifungal proteins in transgenic plants has been demonstrated (Jach *et al.*, 1995; van den Elzen *et al.*, 1993) so the expression of several genes for antifungal proteins is probably required for significantly improved resistance to fungal pathogens.

This work has received generous support from the CRDC for several years. This has allowed the generation of several batches of cotton lines expressing putative antifungal proteins. Several lines with increased chitinase activity (around ten times background levels) and expression of elevated levels of osmotin were generated. We believe that disease resistance is important for improving overall yield under low disease pressure and avoiding yield loss in cases of severe infection. Therefore evaluation of this material that has already been generated was of importance to the cotton industry. The main goal of this project was to generate seed for glasshouse and field testing against Verticillium and Fusarium wilts and to undertake these tests with the best chitinase-expressing material.

Previous work (Regev *et al.*, 1996) demonstrated synergy between a chitinase and Bt toxin against *Spodoptera sp.* It was postulated that this might be mediated by digestion of the chitin framework of the peritrophic membrane. Therefore insect feeding trials to investigate whether chitinase enhances the activity of Bt toxin against *Helicoverpa sp.* were conducted in collaboration with the Division of Entomology.

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

Year 1

(1) Complete assessment of expression levels for constructs not yet characterised (truncated chitinase, truncated osmotin, osmotin, β-1,3-glucanase)

Completed. We have two homozygous lines expressing good levels of chitinase from single gene copies. We found that truncated chitinase lines did not express significantly elevated chitinase levels. One line from those transformed with truncated and full-length osmotin was selected on the basis of mRNA expression. Transformation with a β-1,3-glucanase gene construct has so far yielded 3 putative primary transformants. All 3 expressed the selectable marker gene, NPTII and had higher glucanase activity than an untransformed plant. However, 2 of the plants were male sterile and one was completely sterile. Unfortunately, no evidence of enhanced glucanase expression was detected in the progeny of the two fertile plants. Tissue culture is continuing to generate more glucanase-expressing lines.

(2) Cross chitinase-expressing plants with other transgenic lines and generate F1 seed from these crosses.

The best chitinase-expressing line has been crossed with the best osmotin-expressing line. F3 progeny from this cross have been screened and a double homozygous line identified. The parent chitinase line has also been crossed with V2-Ingard cotton. F3 seed from this cross has been harvested and will be screened shortly to select a double homozygous line for glasshouse testing of Verticillium tolerance.

(3) Generate seed material for testing of chitinase lines against Verticillium and Alternaria. Fine-tune procedures for culture and infection with Alternaria. Perform growth cabinet and glasshouse trials with Verticillium and Alternaria.

Homozygous seed for testing two lines with single chitinase genes and high levels of expression has been generated and glasshouse testing performed. Testing against *Alternaria* has not been performed because of problems with the cabinet used to generate sporulating *Alternaria* cultures.

(4) Perform an in vitro assay using extracts from chitinase-expressing and Bt-expressing cotton leaves in a Helicoverpa diet.

Several assays have been performed by Bill James, CSIRO Entomology. No useful synergy was found.

(5) Cross chitinase-expressing cotton with Bt-expressing cotton for further evaluation of anti-Helicoverpa activity. Select lines expressing both genes.

Performed, see (2) above. Testing of F1 progeny against *Helicoverpa* performed, but no useful effects detected.

Year 2

(1) Generate seed material for testing of osmotin, glucanase and other lines as appropriate against Verticillium and Alternaria. Perform growth cabinet and glasshouse trials with Verticillium and Alternaria.

Homozygous seed for the best osmotin-expressing line has been generated and a glasshouse test performed. Seed for Glucanase-expressing lines not yet obtained (see Year 1(1) above)

(2) Complete glasshouse testing of single gene lines and any multiple gene lines that have been generated.

Glasshouse testing with chitinase and osmotin lines has been performed. Sufficient double homozygous seed from the chitinase/osmotin cross for a glasshouse disease resistance assay has been generated.

(3) If appropriate, generate material for field trials and arrange with Narrabri re conducting of field trials.

GMAC proposal written, field trial with best chitinase-expressing line in progress in *Verticillium* nursery at Narrabri and in the *Fusarium* Nursery at Brookstead in Queensland.

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

Generation of transgenic plants and screening of putative transformed plants was undertaken using standard transformation protocols. Evaluation of chitinase expression was performed using a dye-modified substrate and measuring the release of dye by enzyme activity in plant extracts.

An assessment of the performance of transgenic lines was performed using glasshouse assays inoculated with Verticillium conidia by root dip inoculation. The severity of the subsequent infection was determined using mean plant heights for various lines of cotton and comparing this with the mean plant height for untransformed Coker cotton plants. There are several procedures that may be employed for the assessment of Verticillium wilt, including stem puncture inoculation, and measurement of symptoms on the basis of the extent of vascular browning or leaf necrosis. In this instance it was not possible to discriminate between the transgenic and untransformed lines using symptom-based assessment, but measurement of plant heights gave a small, but consistently observed difference.

Field testing of the chitinase-expressing line was determined using the standard procedures of those staff who undertook the tests, and in accordance with the guidelines agreed to by GMAC.

Detailed results including statistical analysis of results?

Chitinase-expressing plants were consistently taller than untransformed Coker plants after inoculation with Verticillium wilt. This result was observed in several independent trials. Initial trials using osmotin-expressing plants show that these are also less stunted after Verticillium wilt infection. The differences observed are small, but were found to be significantly different (using the t-test) at about 6 weeks after infection.

In the field trial, no differences were observed between the transformed line and untransformed control plants. In the Fusarium trial, almost complete mortality was observed for both lines early in the season.

Assessment of the effects of chitinase in association with Bt was performed using feeding trials with cultures of *Helicoverpa.* Results were variable, but no consistently improved mortality of larvae was observed. Some decrease in weight gain was observed, but consistent results were not obtained.

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

The desired research outcomes compared with objectives were essentially achieved in that seed of a line expressing elevated chitinase levels was generated and the fungal tolerance of this line assessed in the glasshouse and in the field. Unfortunately, the results of the field trial indicate that the levels of protection against stunting observed in the glasshouse was not sufficient to give significant protection against the wilt fungi in the field.

Some aspects of the project goals were not fully met. Work with Alternaria was not completed due to problems with culturing the pathogen, and a decision to focus on the more important vascular wilt pathogens. Generation of plants with demonstrated enhanced glucanase levels has still not been achieved, despite several transformation experiments with two completely independently generated glucanase gene constructs and the use of two different types of enzyme assay to assess the performance of plants. Perhaps there is some underlying reason why cotton does not express elevated glucanase levels.

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

The results with the chitinase-expressing line suggest that expression of this gene does not improve cotton's tolerance to vascular wilt diseases in the field. It is probably unlikely that expression of a just one or two genes for PR proteins (pathogen response proteins) will confer sufficient tolerance to generate useful resistance in the field. In my view, methods for activation and enhancement of the whole suite of defense responses are more likely to be successful in the long term, although they will be harder to achieve.

A description of the project technology (eg commercially significant developments, patents applied for or granted, licences, etc)

na

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

na

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

Completion of assessment of cotton plants expressing elevated levels of both chitinase and osmotin is being undertaken. Evaluation of other transgenic lines generated in this project and by other workers in our laboratory will continue. Generation of transgenic plants transformed with constructs containing more than one PR protein gene has been initiated and seed generation and testing of these lines will continue as material is produced.

A list of publications arising from the research project
What Publications / Published Findings have emanated from the research so far? (Please list)

(1). McFadden, H., de Feyter, R., Murray, F., Grover, A., Llewellyn, D., Dennis, E., and Peacock, W.J. (submitted). Genetic Engineering Approaches to the Improvement of Cotton's Tolerance to *Verticillium* Wilt. **Proceedings, 7th International Verticillium Conference, Greece, Oct 6-10th, 1997.**

(2). McFadden, H. (submitted: invited plenary address). Prospects for Controlling Vascular Wilt Diseases of Cotton and other Crops by Genetic Engineering. **Proceedings, 7th International Verticillium Conference, Greece, Oct 6-10th, 1997.**

(3) Helen McFadden, Anita Grover, Rob de Feyter, Danny Llewellyn and Liz Dennis' Transgenic cotton expressing a gene for Chitinase shows improved tolerance to Verticillium wilt in glasshouse trials. **1998 Australian Cotton Conference Proceedings, 1998.**

(4) H. G. McFadden, G. Lawrence, R. de Feyter, R. Chapple, D. Llewellyn and E. Dennis.
Characterisation of Chitinase Expression Levels in Two Fibre Crop Plants after Pathogen Treatment. Paper in preparation for Physiological and Molecular Plant Pathology, passed for submission by Divisional Editorial Panel.

A one page plain English summary of the project outcomes must be submitted, and this may be used in CRDC publications and on our proposed web site.

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

We have obtained transgenic cotton that makes increased amounts of an enzyme, chitinase, believed to have antifungal properties. These transgenic plants produce about 10 times as much chitinase as their untransformed parents. We tested the tolerance of these transgenic plants to infection by the fungal pathogens that are commonly significant in Australian cotton production, namely *Verticillium* and *Fusarium* wilts. In glasshouse assays we found that the chitinase expressing line was less stunted after *Verticillium* infection than an untransformed control line. Field tests in conditions of high *Verticillium* or *Fusarium* pressure showed no differences between the transgenic and control lines.

Glasshouse testing of an osmotin-expressing line has also shown reduced stunting after *Verticillium* infection. The chitinase and osmotin-expressing lines were crossed and a line homozygous for the expression of these two genes was selected. This line is currently being tested in the glasshouse. Work with other potentially antifungal genes is continuing.

Feeding trials using transgenic chitinase cotton and *Helicoverpa* (bollworm) larvae to test the effects of chitinase on the susceptibility of insects to Bt toxin were conducted. No useful toxicity of chitinase or synergy between chitinase and Bt was found.