Australian Government
Cotton Research and Development Corporation

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

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

CRDC Project Number: **CSP135C**

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:PhD Project- Molecular Biology of Terpene Biosynthesis in Cotton

Project Commencement Date: 22/10/2002 **Project Completion Date:** 22/10/2005

Research Program: 5 Breeding and Biotechnology

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Signature of Research Provider Representative: _____

Part 3.3 – Final Reports (due 3 months after completion of project)

(The points below are to be used as a guideline when completing your final report. Postgraduates please note the instructions outlined at the end of this Section.)

NOTE: This Final Report was requested by CRDC as the project was terminated midstream and no thesis is likely to result.

1. Outline the background to the project.

Seed cotton contributes some $1.3 billion to the Australian economy (Australian Bureau of Statistics 2002; Cotton Research and Development Corporation (CRDC) 2002). While cotton is mainly grown for its lint, the seed is used to produce stock feed, oil and fertilizer.
Cotton (*Gossypium* spp.) is differentiated from other Malvaceae family members by the presence of pigment glands in the foliage, seed and roots. These glands contain a unique group of chemicals including gossypol (G), desoxyhemigossypol (dHG), hemigossypol (HG), hemigossypolone (HGQ), and the heliocides H_1, H_2, H_3, and H_4 (Fig. 1). The dimeric sesquiterpene phytoalexin G is the predominant terpene in the seed and roots. These chemicals play an important role in the constitutive and inducible defence of the plant against a wide variety of pests and microbes. Mutant 'glandless' varieties of cotton (those which contain few (if any) glands) are subject to attack by insects, rodents and birds that are not typically cotton pests (Bottger *et al*. 1964; Lukefahr *et al*. 1966).

The toxicity of free G limits the market value of cottonseed, and must be chemically removed by processing before consumption (Hron *et al*. 1987; Velasquez-Pereira *et al*. 1999). Furthermore, the dark colour of G renders cottonseed oil commercially undesirable, and needs to be corrected by (expensive) refinements (Hron *et al*. 1987; Srivastavas1987). Elimination of G from cottonseed through genetic engineering will enhance the feed value of the meal and reduce the processing costs of cottonseed oil. A variety of transgenic approaches aimed at reducing G levels in the seed without altering the normal protective levels of G in the plant's vegetative parts are currently being investigated (e.g., Townsend 2000; Townsend & Llewellyn 2002; Luo *et al*. 2001).

This PhD project follows on directly from research conducted by Townsend (2000, unpublished data.). The hypothetical sesquiterpene pathway, composed of all enzymes necessary and sufficient for sesquiterpene biosynthesis, is proposed to branch off the central mevalonate pathway (Fig. 1) (reviewed in Chappell 1995). In effect, this means that if any step in this pathway is blocked, G cannot be synthesised. Based on this premise, this research project will investigate the potential transgenic manipulation of seed G levels through three key enzymes of the G biosynthesis pathway: a (+)-δ-cadinene synthase (a sesquiterpene cyclase; CADS); a cytochrome p450 hydroxylase; and a peroxidase. Three putative genes that encode these key enzymes have previously been isolated by Townsend (2000; unpublished data); these genes shall be further characterised and manipulated with a view to modifying the G content of cotton seeds.

CADS catalyses the first committed step in cotton sesquiterpene biosynthesis (Fig. 1). The CADS gene is a member of a large multigene family in cotton (Chen *et al.* 1995; Chen *et al.* 1996; Davis *et al.* 1996; Meng *et al.* 1999; Tan *et al.* 2000). CADS is highly induced in cotton stems (Benedict *et al.*1995; Alchanati *et al.* 1998; Bianchini *et al.* 1999; Tan *et al.* 2000; McFadden 2001) and suspension cultures (Chen *et al.* 1995; Chen *et al.* 1996; Lui *et al.* 1999; McFadden 2001) infected with the pathogen Verticillum wilt, *Verticillum dahliae* Kleb., and in cotyledons infected with the bacterial blight pathogen, *Xanthomonas campestris* pv. *Malvacearum* (Smith) Dye (*Xcm*) (Chen *et al*. 1995; Davis & Essenberg 1995; Davis *et*

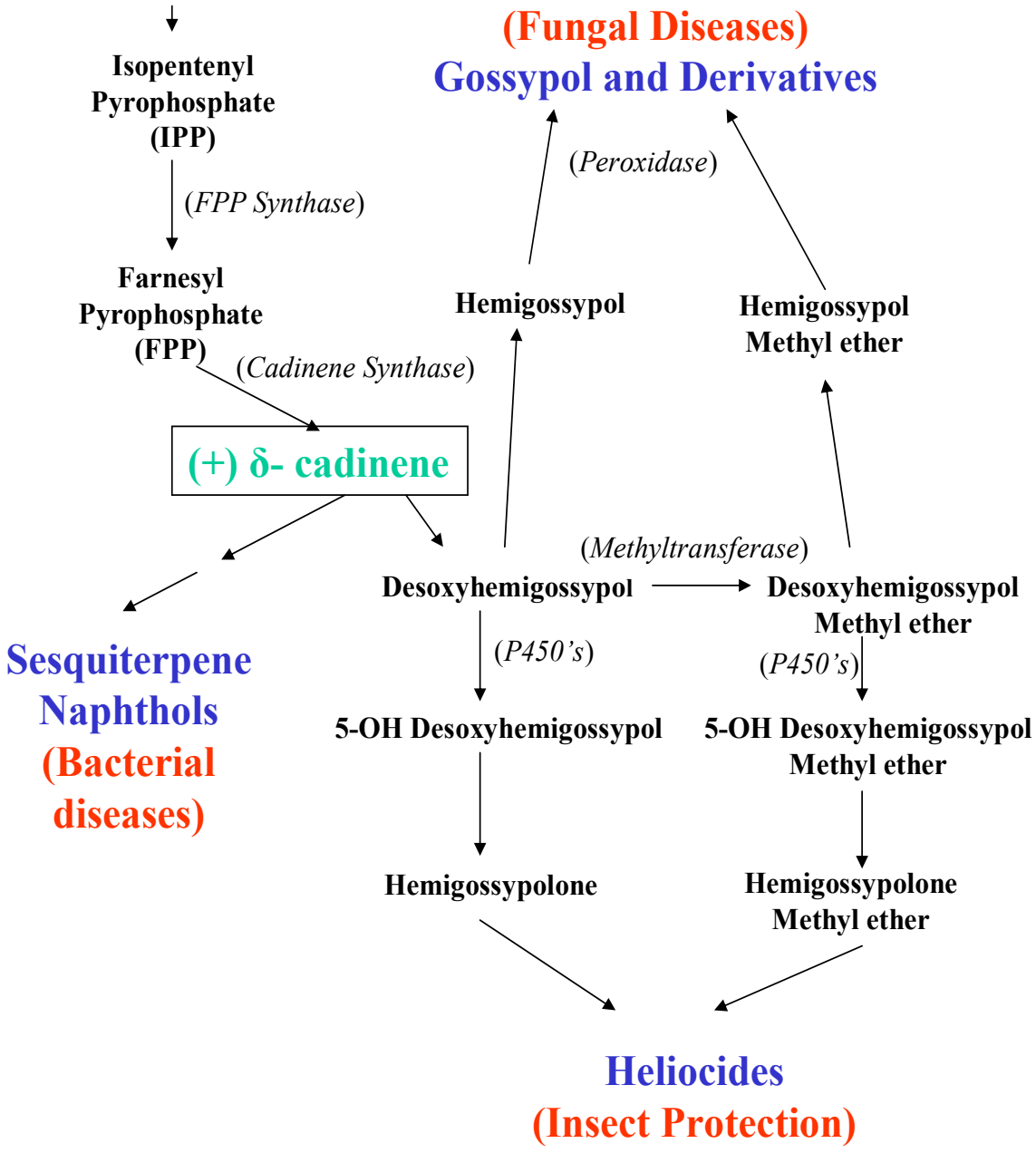

Figure 1. Simplified biosynthetic pathway for cotton defence terpenes (key enzymes are in italicised in brackets)

al. 1996). Townsend (2000) isolated from *G. hirsutum* a nearly full-length CADS cDNA clone (C6, *cdn*2: AF270425) highly homologous to a CADS gene isolated from *G. arboreum* (Chen *et al.* 1995) and to terpene cyclases from other plant species. Plants transformed with cotton antisense (+)-δ-cadinene synthase gene constructs and subjected to infection experiments exhibited an altered defence response against Bacterial Blight infection, but not

Verticillum Wilt. G levels in the seed remained constant (Townsend, unpublished data). These results may indicate differential multigene family member expression patterns. Further analysis suggests that this gene may not be directly involved in biosynthesis of G but rather sesquiterpene napthols (Townsend 2000, unpublished data).

Townsend (2000, unpublished data) further isolated two apparently functional genomic CADS clones: *cdn*-GC2 (the genomic equivalent of C6) and *cdn*-GC4 (a putative seed-specific CADS-encoding gene). Translational fusion of the promoter sequences from each gene to the β-glucuronidase (GUS) reporter gene allowed preliminary assessment of promoter activity in embryonic tissue. Initial analysis of GUS activity in embryogenic callus and differentiated embryos indicated that the *cdn*-GC4 promoter activates gene expression in young leaves and stems and not in undifferentiated callus tissue, while the *cdn*-GC2 promoter appears to operate in the opposite fashion. Further characterisation of the GC4 construct will be carried out to confirm that it is from an embryo-specific form of CADS.

It is hypothesised that several steps in the gossypol biosynthesis pathway are catalysed by cytochrome P450s, a large superfamily of proteins ubiquitous in prokaryotes and eukaryotes. Townsend (2000, unpublished data) isolated a *G. hirsutum* partial cDNA putative cytochrome P450 clone (P2) with a high degree of similarity to other P450 genes involved in terpenoid biosynthesis. A synthetic full-length clone was consequently constructed. Expression analysis indicates that this P450 is transcriptionally induced by infection with *Xcm*, although it has not been shown to have a direct role in the biosynthesis of gossypol. Further analysis will be carried out to try to identify the biochemical step catalysed by this P450 enzyme.

It is believed that a peroxidase catalyses the final step in gossypol production. Townsend (unpublished data) isolated a full-length synthetic copy of a *G. hirsutum* putative peroxidase gene that showed high similarity to peroxidase genes other than those involved in lignification in other plants. Preliminary RT-PCR analysis indicated that expression of this peroxidase is high in roots and embryos, suggesting a possible role in gossypol biosynthesis. Expression is also induced in stems in response to *V. dahliae* infection. Two genomic clones have since been analysed, and show evidence of differential splicing of pre-mRNA transcripts under different conditions. Again further experiments will be carried out to identify the exact biochemical step catalysed by this peroxidase enzyme.

Once we are satisfied with the importance of the different enzymes in gossypol synthesis, new gene constructs will be made to try to manipulate the expression of these enzymes in seeds to see if we can reduce the amount of gossypol made in seeds without affecting terpene synthesis elsewhere in the plant.

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

The objectives of this research project are to gain insight into the sesquiterpene biosynthesis pathway, especially in relation to regulation of its key genes, and to consequently produce (or

enable production of) transgenic cotton plants with G-free cottonseed and normal G levels in the plant's vegetative parts. This will permit the production of cottonseed safe for animal and human consumption without significantly altering the insect resistance of the cotton plants.

Major project objectives include:

1. Characterisation of the genomic cadinene synthase clone GC4 isolated by Townsend (2000) that may be responsible for seed gossypol production
2. Sequencing the remaining cadinene synthase genomic clones isolated by Townsend (2000) to determine whether they express seed-specific cadinene synthase
3. Isolation of novel, seed-specific cadinene synthase genes
4. Characterisation of the properties of the cytochrome P450 (P2) isolated by Townsend (2000)
5. Isolation of novel P450 genes from cotton that may regulate gossypol biosynthesis
6. Characterisation of the peroxidase gene isolated by Townsend (unpublished data)

3. Detail the methodology and justify the methodology used.

One of the objectives of the project is to produce plants that make gossypol and its intermediates in all of the normal tissues of the plant except for the seed where it reduces the nutritional value or increases processing costs of seed meal and oil. The established way of knocking out or silencing a gene is to use the hair-pin or RNAi technology whereby an introduced gene or fragment of a gene in a sense and anti-sense orientation results in the production of a double-stranded segment of RNA which in turn induces a defense response causing the sequence specific degradation of single stranded RNA matching the double stranded RNA coming from the introduced construct (Wang and Waterhouse, 2002). This technology has been used previously in cotton to silence a gene involved in oil desaturation and produce a plant with a different oil composition (Liu et al., 2000). We have used this technique to try to knock out the expression of two different types of genes; a P450 hydroxylase and a peroxidase gene both isolated from cotton. Transgenic plants have been produced and now need to be analysed at the molecular level to demonstrate the presence and expression of the introduced genes constructs and at a biochemical level to see what \effects this has on terpene composition. The biochemical analysis is outside our expertise so we have been collaborating with natural product chemists at the ANU to seek their participation and advice on analytical methods. The Research School of Chemistry and the Chemistry Department have both made available HPLC and GC-MS equipment to use in the project and have provided training for the postgraduate student working on the project and we hope that this collaboration will continue in the future. CSIRO has its own Analytical Services unit and GC-MS equipment that can also be used in the project.

The analysis of expression patterns of specific genes can be carried out using a variety of molecular techniques including Northern blotting, in situ hybridisation and RT-PCR but for the examination of tissue specificity one of the best techniques is to isolate the promoter of the particular gene and link it to a reporter gene (Such as GUS or GFP) and introduce the novel construct into transgenic plants. Detailed microscopic examination (with or without staining, depending on the marker gene used) can then be carried out to examine the cellular or tissue specific patterns of expression driven by the reporter genes. This has been used with the GC4 Cadinene Synthase gene promoter being studied here and has indicated that the promoter is embryo-specific. A similar strategy was used to examine the expression patterns of a heterologous gene promoter, the soybean lectin promoter used in previous experiments by Belinda Townsend.

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

1. One of the first components of the projects was to begin the analysis of the promoters of one of the Cadinene synthase promoters (GC4) isolated by Belinda Townsend. This promoter had been isolated from a genomic segment of cotton and linked to the GUS reporter gene so that when introduced into transgenic plants the pattern of gene activity specified by the promoter could be determined by following where the GUS reporter enzyme was expressed in the plant. This is normally done qualitatively by staining different plant tissues with a specific substrate for the GUS enzyme (X-Gluc) and looking for the intense blue colour reaction that indicates presence of the enzyme in particular tissues. Four different transgenic lines had been generated with the GC4-GUS construct and preliminary analysis was carried out on the T1 seed from these plants. This involved planting seeds, isolating DNA from different individuals and testing fort the presence of either GUS marker or the linked kanamycin resistance gene (NptII) using PCR to determine which plants were transgenic. Different tissues (leaves, stems, roots, flowers, seeds) of these plants were then incubated with X-Gluc stain overnight and then destained in an ethanol series and photographed (Fig 2.).

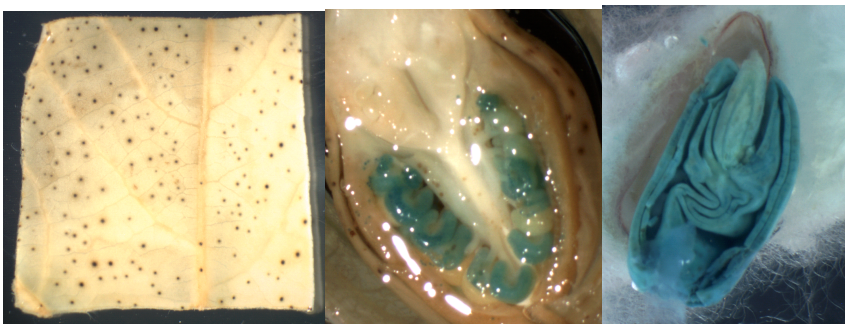

Figure 2. X-Gluc staining (left to right) of cotton leaves and young and relatively mature seeds. GC4-GUS is expressed only in the embryo at different ages.

While more extensive analysis is needed, particularly at different ages of seeds, it would appear the GC4 promoter drives what appears to be high expression in the embryo. It is not expressed in the leaves, stems (not shown), roots or in surrounding tissues of the boll. There appeared to be low expression in the fibre but this may be an artefact and needs to be confirmed. We need to look at the next generation to confirm this pattern of expression but at this stage the GC4 promoter may provide us with a good embryo specific promoter. It is interesting to not that we did not see gossypol gland-specific expression of the GC4 promoter and may suggest that gossypol is synthesised throughout the embryo but translocated into the gossypol glands as they develop. Detailed microscopic analysis needs to be carried out but it looks as if this gene is expressed strongly in the gland tissues as well as other tissues of the cotyledons. It is possible that other CADS genes may confer gland- specific expression. A second CADS gene GC2 was also isolated and we have four lines containing a GC2-GUS

fusion gene which are yet to be analysed, but staining at the embryogenic callus stage did not suggest that this gene was embryo-specific so may be very different from GC4. We need to look at the induction of expression of both constructs during pathogen infection to see if they have different functional roles. This would make sense as cotton has over 14 different CADS genes and we would expect this diversity was driven by them having different functions in chemical defense.

2. As yet the characterisation of some of the other CADS genes originally isolated by Belinda Townsend in her PhD project has not been started, but the clones have been safely stored in the freezer until this can be carried out.

3. This was an option if the analysis of the GC4 CADS gene did not show that it was embryo-specific. At this stage the GC4 gene looks to be a useful target for gene knock out so other embryo-specific genes may not be required.

4. Townsend (2000) isolated a novel P450 gene (P2) from cotton that appeared to be induced by pathogen infection. As plants can contain hundreds of different P450 genes it was important to verify that this gene was in deed involved in the gossypol biosynthetic pathway and not some other pathway. The cotton gene was related to the CYP71B class of P450 genes. We had two options for this, one using transgenic plants and one using transgenic yeast. Progress towards both systems had been made but as yet no definitive answers are available. The plant system relied on generating a gene silencing construct specific for the P450 hydroxylase and transforming it into transgenic cotton. This should result in efficient silencing or suppression of the gene and hence the failure of the plants to carryout the enzymatic step carried out by the P2 gene product. The transformation work was being carried out by technical staff employed by the Core Biotechnology project but the analysis of these plants was being undertaken as part of the PhD project. Several transgenic lines have been produced so far and these are being characterised at a molecular level to determine that they contain the transgene (PCR and Southern blotting) and taken through to the T2 generation before any extensive biochemical analysis. T2 seed of a number of lines has been generated and is ready for analysis of the terpene profile by HPLC, GM-MS or other techniques. Unfortunately the student involved resigned suddenly and this analysis has yet to occur.

The second strategy to identify a function for the P450 gene is to express it yeast and feed the yeast various precursors of terpene synthesis and chemical analyse the biochemical conversions. A similar strategy has been used to characterise other P450 genes, including the cadinene 8-hydroxylase (CYP706A type) recently isolated by a Chinese group that catalyses possibly an earlier step that our P450. We have been able to obtain the Chinese P450 construct and can use it as a control in our own yeast experiments and perhaps even co-express it with our gene to see if they are both needed to produce the next intermediates in gossypol biosynthesis. One of the difficulties encountered with this strategy was that the main gossypol precursor, delta cadinene, which was available commercially a couple of years ago has been taken off the market so can no longer be purchased. Saara Bowen was investigating alternative sources of this chemical and in collaboration with scientists at the Research School of Chemistry (ANU) had sourced a number of essential oils (cade oil, teatree oil) that are relatively rich in delta-cadinene and was starting to extract it from those oils by spinning band steam distillation and other organic chemistry methods. It was concluded that delta-cadinene could be isolated in this way but it would need time dedicated to the extraction. We hope to have a part-time graduate Diploma student work on this project later in the year so they should be able to complete the delta-cadinene extraction and the yeast bioconversion experiment.

5. This was proposed as a back-up experiment if the P2 P450 gene did not prove to be involved in gossypol synthesis but some other biochemical pathway and would have been carried out in a similar way to the original P2 gene. Cotton is expected to have a couple of hundred P450 type genes and they are best identified in sort of differential screening like that used for the P2 gene where P450 gene sequences that were expressed in pathogen induced tissues were compared to those in healthy tissue to pick the ones specific for the disease response and hence likely to be a gene involved in terpene phytoalexin production.

6. The peroxidase isolated from pathogen infected cotton (Pox5) was unusual and differed from other peroxidases known to be involved in cell wall lignification during pathogen infection, but was similar to pathogen induced peroxidases of unknown function in other species. Southern hybridisation revealed the gene to be a single copy in the diploid species. Expression of this peroxidase gene is high in roots and embryos, which correlates well with a possible role in gossypol biosynthesis. Expression is also induced in stems in response to Verticillium infection, an unexpected result since gossypol levels are reported to be low in stem xylem. Several genomic clones have been isolated and two individual representatives have been analysed so far. A comparison of cDNA clones and the genomic clones reveals that differential splicing of pre-mRNA transcripts may occur. This is an interesting result that could represent a different level of gene regulation in response to different stimuli. Inverted repeat gene silencing constructs have been made and transformed into cotton by the Core Biotech Program technicians. These plants are becoming available and we have started to characterize them and produce homozygous T2 seed for proper biochemical analysis as for the P2 silenced plants in section 4. A full length representative of this gene was under construction for heterologous protein expression and analysis of the enzyme activity in bacteria (peroxidase can be easily expressed in bacteria and we do not have to resort to the use of yeast as an expression system), but this has yet to be completed.

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

The project was well down the track when the student on the project had to resign for her own personal family reasons. This was disappointing as there were many things still to be completed in the project. A good foundation had been set up with the analysis of a number of different types of transgenic cotton plants to look at the tissue-specificity of one specific cadinene synthase gene that appears to be seed or embryo-specific and hence will be useful to target our gene silencing constructs to the tissues where gossypol is being made. We had two other potential targets for silencing, a P450 gene and a peroxidase gene but we needed to gather evidence that these were involved in gossypol production and not some other defense chemical, The material is all now available but we need someone with good analytical chemical skills to complete the analysis and then develop new gene constructs that could be used to turn off gossypol production in seeds.

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

The aims of this project were three fold: to provide training for a postgraduate student in a variety of molecular and biochemical techniques so that they will be able to contribute to cotton science and the Industry as an independent researchers at a later date; to increase our knowledge of the biochemistry and molecular biology of the production of secondary chemicals in cotton plants; and putting these together, to try to genetically modify cotton so that it makes these chemicals in the vegetative parts of the plant but not in the seeds where they reduce the economic value of cottonseed as a animal feed and contaminate oil processed

from cottonseed. Processed oil must be extracted with harmful organic solvents to remove gossypol and other terpenes adding to costs of production and creates problems of disposal of the by-products.

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 generated transgenic cotton expressing a reporter gene (GUS) driven by the cotton cadinene synthase 4 promoter that appears to be embryo-specific. This promoter will be useful for targeting transgenes to the cells that are making gossypol and its related chemicals so i\s a new tool for metabolic engineering in cotton. This promoter appears to be expressed in all the cells of the developing cotton cotyledon, not just the gossypol glands and suggests that these sesquiterpenes are not made just in the gland cells but all through the embryo and are then transported and stored in the glands.

We have cloned and partially characterised two other types of genes, a P450 hydroxylase and a peroxidase that may be involved in gossypol synthesis but this still needs to be verified. These are all possible targets for silencing in seeds to prevent gossypol production but will require a number of years more work before this could be achieved.

No changes are required to the IP register.

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 project was suddenly terminated in the middle with the resignation of the postgraduate student working on the project so there are still many things that were only partially completed. We have continued to grow and analyses some of the transgenic plants produced for the project but this will only be as time and resources allow. One aspect of the project, to identify the biochemical function of the P450 gene may be taken up by a graduate Diploma student but this is currently being worked out.

As and when aspects of the project are completed they will be written up for publication in reputable scientific Journals. The project (undeservedly, I think) did not receive a high recommendation for funding by the recent Biotech and Breeding Review Committee because similar work has been going on unsuccessfully in the US for the last ten years but this puts too much emphasis on the applied outcomes of the project and does not take into account the training function of Postgraduate projects. There are still many things to be discovered about how plants regulate the synthesis of the multitude of different defence chemicals produced in response to pathogens and pests and such project cover all aspects of plant molecular biology, biochemistry and genetic engineering so provide perfect training grounds for young plant scientists. For the moment, however, we would propose to continue the project internally to complete some of the more advanced aspects of the research to the stage of publication. We

do not anticipate applying for a new Scholarship in the coming round as proposed building refurbishments over the next year will make it difficult for us to house a new student. In retrospect, our previous student on this project was probably marginal in her performance (although she did improve with time) and highlights the importance of finding the best candidates for these positions even if it takes a little longer as a lot of other staff time and energy needs to be committed to every new student.

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

No Publications at this stage as none of the project objectives were completed due to the sudden and unexpected resignation of the student involved.

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

No

11. 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.

At this stage there are a number of unfinished experiments that will need to be completed before we can anticipate any benefit to the Australian Cotton Industry. The project was still at an early stage and its main objectives were to look at a couple of potential new targets for genetic manipulation that might result in plants with less gossypol in their seeds and hence increase the value of the seed for both human and animal consumption.

POSTGRADUATE SCHOLARSHIPS are not required to submit a final report. Recipients are to send a statement from their supervisor confirming that their thesis has been submitted for assessment. Following examination and any necessary correction, a single copy of the thesis is to be forwarded to CRDC, along with an electronic copy of the Plain English Summary.

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.

This project aimed to investigate the expression and function of a number of genes that we believed were involved in the production of the secondary chemical gossypol in cotton. One of the key enzymes in cotton terpene synthesis is cadinene synthase that is encoded by several different members of a large gene family. The promoter of one of these genes was chosen for analysis based on it sequence and when linked to a reporter gene and inserted into cotton drove expression of that GUS reporter gene in an embryo-specific pattern. As we were after a promoter that would allow us to silence gossypol production in cotton seeds (ie embryos) but not elsewhere in the plant this now provides us with a useful molecular tool for delivering gene knockout constructs to the cells in the seed that are making gossypol.

In addition we had previously isolated a gene for an enzyme of the P450 hydroxylase class that are involved in many biochemical conversions of secondary defence chemicals, but we needed to demonstrate that it was involved in gossypol production not some other chemical in cotton. Gene silencing or knockout constructs have been developed for this gene and introduced into transgenic cotton. We hope to be able to analyse the chemical composition of the oils in the gossypol glands to see what effects plants that should be deficient in the hydroxylase have had in terms of terpene production. In an alternative strategy we are also expressing this gene in simple bread yeast to see what biochemical conversions it can achieve when fed with different intermediates in gossypol synthesis.

A third approach has been to clone a peroxidase we believe is involved in the last step of gossypol production and see what changes in chemical composition occur when this particular gene is silenced in transgenic cotton plants.

Overall we are trying to characterise some of the key enzymes and genes in gossypol synthesis and develop strategies for eliminating gossypol just from the seeds of transgenic cotton plants in an attempt to increase the nutritive value of cotton seed meal and oil without affecting the normal defensive role of these chemicals against pests and pathogens of cotton. Unfortunately, the project was suddenly terminated midstream by the resignation of the student for personal family reasons.