January, August & Final Reports

REPORTS

Part 1 - Summary Details

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

CRDC Project Number: **CSP105C**

January Report:	☐	Due 29-Jan-02
August Report:	☐	Due 02-Aug-02
Final Report:	☒	Due within 3 months of project completion

Project Title: Potential for the genetic manipulation of gossypol - a defence chemical with negative impacts on cottonseed products

Project Commencement Date: 12/7/1999 **Project Completion Date:** 11/7/2002

Research Program: Plant Breeding and Biotechnology

Part 2 – Contact Details

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Signature of Research Provider Representative: ____________

Part 3 – Final Report Format

1. Outline the background to the project.

Gossypol is a naturally occurring sesquiterpene chemical and a product of secondary metabolism unique to cotton species. Gossypol is important to the cotton plant because it provides a degree of natural resistance against pests and diseases. Gossypol is the end product of just one branch of sesquiterpene biosynthesis in cotton, with other branches leading to the antibacterial phytoalexins of the lacinilene group, and the important pest protection chemicals of the heliocides. It and its derivatives are stored in the gossypol glands spread throughout the plant as well as being induced in other tissues when the plants are attacked by disease organisms. Unfortunately gossypol is toxic to humans and monogastric animals, and cottonseed products must undergo expensive post-harvest treatments to remove the high levels of gossypol from oil and meal before consumption. The ideal cotton plant would possess high levels of gossypol in the plant and negligible levels in the seed. This is a characteristic already present in the native Australian cotton species, *Gossypium sturtianum*, however has proven exceedingly difficult to introgress this trait into cultivated species by traditional breeding methods. Genetic engineering offers another way of producing this phenotype if we have a clear understanding of the enzymes and genes responsible for gossypol production. This project aimed to clone some of the important genes in gossypol biosynthesis and use them in transgenic plants to specifically reduce gossypol production in cotton seeds.

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

The broad objectives of the project were therefore to investigate the molecular processes controlling the biosynthesis of sesquiterpene phytoalexins or defence chemicals in cotton. These phytoalexins included gossypol, lacinilene C, desoxyhemigossypol, and heliocides as examples. Specific genes involved in the pathway were targeted for cloning, characterisation of gene expression patterns in different plant parts as well as the control of their expression in pathogen challenged cotton plants.

Two genes were isolated from cotton that were induced by infection with a bacterial pathogen – implicating them as having an involvement in the disease response. One of these genes, cadinene synthase, is the first committed step in gossypol biosynthesis and a good target for blocking the entire pathway using genetic engineering approaches. The other gene, a cytochrome P450, may be responsible for downstream hydroxylation reactions in the pathway but a biochemical function has not been determined for this gene yet. The foundations have been laid from which to build a more comprehensive understanding of gossypol biosynthesis at the molecular level.

3. How has your research addressed the Corporations three outputs: Sustainability, profitability and international competitiveness, and/or people and community?

Genetic engineering of cotton has the potential to improve several aspects of cotton production ranging from improved environmental sustainability to streamlined processing applications. With the background work undertaken as part of this project we are a step closer to reducing gossypol levels in the seed which would achieve a more sustainable and profitable industry due to reduced chemical usage and processing requirements for the use of the cotton seed for oil and meal. The people involved in cotton production could also benefit by a reduced amount of lacinilene C (a possible side effect of reducing gossypol) in dried bract tissue that is known to be a contributing factor of byssinosis in cotton mill workers.

4. Detail the methodology and justify the methodology used.

The project utilised standard molecular biology techniques, biochemistry methods, and modern genetic engineering approaches to investigate the gossypol biosynthetic pathway and to try and generate industrially useful engineered cotton plants. Reverse transcription polymerase chain reaction (RT-PCR) and conventional PCR was employed for expression analysis and gene cloning. Gene library screening was used to isolate the cDNA and genomic clones of interest. Hybridisation methods were used for expression analysis and assessing gene copy number and transgene inheritance. Gene construct generation was an important aspect of the genetic engineering side of the project as well as the associated analysis of a heterologous seed specific promoter. Other methods such as database mining and computer aided analysis of the phylogenetic relatedness of genes of interest were also important as a way of gaining more information about the genes studied. More classical biochemical approaches like heterologous protein expression, NMR, HPLC, and GC-MS, proved critical in confirming enzymatic function of the gene products. Antibodies were generated using purified recombinant protein. Naturally occurring mutants of cotton that lack the gossypol glands associated with pest tolerance were also useful tools in the analysis of genes of interest and to further our understanding of the regulation of induced defence responses in cotton.

5. Detail results including the statistical analysis of results.

A cDNA clone encoding (+)-δ-cadinene synthase (CADS) was identified from *Gossypium hirsutum*, as well as its genomic gene equivalent and several other closely related CADS genes that form part of a large multigene family in cotton. The existence of this multigene family complicated our attempts to manipulate gossypol biosynthesis but we postulate it to be important for the differential regulation of secondary defence chemical production in cotton in response to various pathogen challenges, stress responses and normal growth. The generation of transgenic cotton plants designed to block expression of CADS in the seed or the whole plant did not produce an expected downstream effect of a reduction in seed gossypol levels, although there was a reduction in the induction of CADS enzyme and mRNA levels when transgenic plants were challenged with a bacterial pathogen (but not with a fungal pathogen). Given the level of sequence homology between the different CADS genes we had expected our antisense constructs to silence many of the different classes of CADS genes but it appears we have only been able to affect the CADS genes involved in the bacterial defence response. Seed specific CADS may have been inhibited but other non-silenced CADS genes may have compensated for the reduction in activity of any specific members of the CADS gene family. Further research will be needed to clarify this. The CADS gene we isolated from seeds (C6) was recombinantly expressed in the bacterium *Escherichia coli*, purified to homogeneity and used for the generation of antibodies, enzyme assays, and enzyme product identification. This data confirmed that the cloned gene did encode (+)-δ-cadinene synthase by analysis of the enzyme product produced in vitro from farnesyl pyrophosphate as the substrate using NMR, GC-MS and chiral GC-MS.

As part of the research into the potential for manipulating gossypol biosynthesis in cotton via genetic engineering, we examined the expression pattern in cotton of a seed specific gene promoter from the soybean lectin gene using a reporter gene (GUS) to ensure that the promoter was active where required in the cotyledons and the cells surrounding the developing gossypol glands. This experiment involved the generation of extra gene constructs with the soybean lectin promoter fused to the GUS gene and the assessment of histochemical staining for GUS across the plant as a measure of promoter activity. It was found that this promoter was ideal for our purposes, being expressed in all the tissues of interest, namely the developing cotyledons of the seed and their immature and mature gossypol gland cells, as well as being active at the correct times, that is before the deposition of gossypol occurs within the glands of the developing seed and including the period of seed germination.

A cDNA clone and the corresponding partial genomic clone of a cytochrome P450 gene were also isolated from *G. hirsutum*. The low expression levels of this gene proved difficult for expression analysis but RT-PCR results did show an apparent upregulation of transcript in response to infection with the Bacterial Blight pathogen. The P450 gene has been inserted into specialised gene constructs for expression of the recombinant protein yeast. Constructs have also been generated for silencing of this gene in cotton, an experiment designed to alter the metabolic profile of the resulting transformed cotton plants and hopefully provide clues as to the biochemical function of this gene – thus providing evidence for or against a role in gossypol biosynthesis.

A new aspect of the project at the very early stage was the aim of isolating a gene encoding ocimene/myrcene synthase from cotton. Ocimene and myrcene are important chemicals for heliocide biosynthesis when these monoterpenes react with gossypol. Phylogenetic analysis of terpene cyclase genes related to CADS revealed a very high level of clustering of relatedness that coincided with function and substrate/product relationships. Using this information we generated a gene probe from an *Arabidopsis thaliana* ocimene/myrcene gene sequence that will be used to screen a cotton gene library to find a cotton homologue of this gene. The gene library to be screened will be a cDNA library made from leaves late in the growing season when ocimene and myrcene levels are highest. This library was constructed by Dr Emmanuelle Faivre-Nitsche as part of a CRDC funded project (CSP102C). A cotton ocimene/myrcene synthase will provide another genetic tool for manipulating heliocide biosynthesis in cotton and may allow us to improve insect tolerance.

6. Discuss the results, and include an analysis of research outcomes compared with objectives.

The isolation and analysis of expression of the genes encoding CADS and a cytochrome P450 from cotton were important steps towards characterising the gossypol biosynthetic pathway at the molecular, and to some extent the biochemical level. The primary goal of this project was to investigate the potential for manipulating gossypol biosynthesis in cotton for improved industrial uses. To a large extent this goal was achieved despite the unsuccessful first attempts at generating gossypol-free cottonseed. What we did learn was that there are numerous steps in the pathway that could be manipulated, and that complicating factors such as gene families and cross-talk between their members may be issues that need more consideration in gene construct design than first thought. This type of conclusion has become common in the field of secondary metabolism and researchers in this area increasingly have to look beyond the "single gene-single phenotype" mentality to achieve their goals. Future work will look at the possibility of using other genes that occur as single copies in the cotton genome and at later stages of the pathway for manipulating gossypol metabolism. In addition new technologies for switching off or silencing specific genes or classes of genes have been developed at CSIRO Plant Industry and this will make specific silencing of members of the gossypol family of biosynthetic genes more efficient and improve our chances of generating useful transgenic products.

7. **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 and future research needs.**

As expected with any genetic engineering based project, because of the long timeframes for development and commercialisation, the results of this project will not have any immediate impact on the cotton industry. However, the research has furthered our understanding of the molecular mechanisms governing gossypol and secondary metabolite biosynthesis in cotton and thus brings us closer to being able to manipulate the pathway for reduced gossypol levels in seed setting up a solid framework for further research by a newly appointed PhD student.

8. **Describe the project technology (eg. commercially significant developments, patents applied for or granted licenses etc).**

No commercially significant developments at this stage although the cytochrome P450 gene analysis and ocimene/myrcene gene isolation expected in future research into this project could provide valuable intellectual property.

9. **Provide a technical summary of any other information developed as part of the research project. Include discoveries in methodology, equipment design, etc.**

No extra technical details other than those supplied above.

10. **Detail a plan for the activities or other steps that may be taken;**

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

A CRDC grant for a postgraduate studentship has been awarded to continue this project. The student (Ms Saara Bowen) will be in a good position to follow up on many aspects of the project that remain to be studied. In particular this includes the cloning of a cotton ocimene/myrcene synthase gene that may be a useful tool for improved pest resistance, characterisation of the cytochrome P450 and new generation CADS transformed cotton plants currently undergoing the tissue culture process, functional characterisation of the cytochrome P450 enzyme, and dissection of the CADS multigene family, as well as the possible target of any new genes of interest involved in the gossypol pathway.

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

The current state of the research should facilitate the publication of scientific manuscripts within a reasonable time frame because several aspects need only final experiments to finalise the data.

11. List the publications arising from the research project.

Townsend, B.J. and Llewellyn, D. J. (2002) Spatial and temporal regulation of a soybean (*Glycine max*) lectin promoter in transgenic cotton (*Gossypium hirsutum*). *Functional Plant Biology* (*Formerly Australian Journal of Plant Physiology*) **29** (7) pp. 835-843

Townsend, B and Llewellyn, D (2002) Designer plants: Manipulating cotton defence chemicals. *The Australian Cottongrower* **23** (1) pp.66-70

Townsend, B. and Llewellyn, D. (2001) Potential for the genetic manipulation of gossypol in cotton – a defence chemical with negative impacts on cottonseed products. In: *Plant foods for human health – Manipulating plant metabolism to enhance nutritional quality*. Breckenridge, USA, p. 56

Townsend, B. J. (2000) Molecular biology of gossypol biosynthesis in cotton. PhD thesis, Faculty of Science and Engineering, The Australian National University, Canberra, Australia. 237 p.

Townsend, B. and Llewellyn, D. (1999) (+)-□-Cadinene synthase expression patterns in bacterial blight infected cotton cotyledons. In: *Interactions and intersections in plant signaling pathways* – Keystone symposia on molecular and cellular biology. Coeur d'Alene, USA, p. 54

Townsend, Belinda (1998) Investing in the industry's future – Molecular biology of gossypol biosynthesis in cotton. In: *Spotlight on cotton research - CRDC activities 1998*, Cotton Research & Development Corporation p. 48

Townsend, B and Llewellyn, D (1996) Molecular biology of gossypol biosynthesis in cotton In: *Cotton on to the future* - Proceedings of the eighth Australian cotton conference, Broadbeach, Queensland. The Australian cotton growers research association Inc., pp. 607-610

12. Are changes to the Intellectual Property register required?

No changes required.