Final Report for ANU3C

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

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

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Molecular Biology of Gossypol Biosynthesis in Cotton

CRDC Project Code
ANU3C

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Final Report

What was the background of the project?

Gossypol is a naturally occurring 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 of cotton. Unfortunately gossypol is toxic to humans and monogastric animals, and cotton seed products must undergo expensive post-harvest treatments to remove the high levels of gossypol from oil and meal for consumption. The ideal cotton plant would posses 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 it is exceedingly difficult to introgress this trait into cultivated species by traditional breeding methods.

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

This study used molecular biology approaches to investigate the biosynthesis of gossypol at the genetic level. Our objectives were to isolate some of the genes involved in gossypol biosynthesis, to characterise their expression and importance in the pathway, and to use these genes to generate transgenic cotton plants with altered levels of gossypol or other terpenoid aldehydes.

In the course of this project two genes were isolated, sequenced and characterised. One gene was particularly exciting as a target for the manipulation of gossypol levels. This gene was used in gene constructs for the generation of transgenic cotton plants. The transgenic plants did not show altered gossypol levels in the experiments carried out so far, but it was demonstrated that the expression of this gene could be altered. This result alluded to the idea that it may still be possible to use this gene to manipulate terpenoid biosynthesis, but that a different variant of this original clone may have to be used in the future.

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

Standard molecular biology techniques were used in this project along with some biochemical and chemical techniques. These included the polymerase chain reaction, gene cloning, gene library screening, DNA sequencing, Northern, Southern and Western blotting, protein expression and purification, generation of antibodies, cotton transformation and analysis of transformants, enzyme assays and HPLC. Cotton plants were also routinely grown under controlled glasshouse conditions.

Detailed results including statistical analysis of results?

Two genes were isolated from cotton during the course of this work. The first encoded a Cytochrome P450 enzyme, predicted to act at an important branch point in gossypol biosynthesis. The expression of this gene has proven difficult to characterise due to low expression levels, but work is continuing on this aspect. It is suspected that this particular Cytochrome P450 gene from cotton may not play a direct role in gossypol biosynthesis. There are predicted to be hundreds of Cytochrome P450 genes in cotton and efforts will continue into the isolation of one involved in gossypol biosynthesis specifically.

The second gene cloned was cadinene synthase, which has been demonstrated to be a key step in regulation of the entire gossypol pathway. The expression of this gene was characterised at the transcript level and to some extent the protein level. This gene was found to be strongly upregulated in response to infection with Bacterial Blight and was therefore likely to play a key role in the plants defence response. This gene was inserted into gene constructs for the tranformation of cotton.

Three cadinene synthase gene constructs were made. One construct directed the seed specific antisense expression of cadinene synthase - designed to block gossypol biosynthesis in the seed only. This employed a Soybean lectin promoter known to direct seed specific expression in other species but it had not been tested in cotton before. Therefore this promoter was used to drive the expression of the GUS reporter gene to test the activity of the promoter in cotton. Results so far indicate this promoter was a good choice for seed specific expression in cotton because it appears to be active early in seed development and at a high level throughout seed development. Unfortunately, the seed specific antisense expression of cadinene synthase (C6 clone) did not seem to reduce the levels of gossypol in the seed (preliminary data so far).

The third construct directed the constitutive antisense expression of cadinene synthase throughout all tissues and developmental stages. At first analysis this did not seem to alter gossypol levels. However after induction with Bacterial Blight these plants demonstrated a lack of induction of cadinene synthase transcript and protein compared to the control, but HPLC data of gossypol levels showed no significant difference. This was an important result for this project and demonstrated that cadinene synthase levels can be manipulated using transgenic plants. It will be important in the future to monitor changes in the levels of compounds related to gossypol (such as the sesquiterpene napthols), as well as gossypol itself, to determine if altered cadinene synthase levels result in the alteration of a suite of chemicals, besides gossypol.

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

The cloning of cadinene synthase and its identification as a key gene in the gossypol biosynthetic pathway represented an important first step in achieving the objectives of this project. Its use in generating transgenic cotton plants was a labour intensive process that consumed much of the project time. Transgenic plants were successfully generated, however of the two constructs, neither appeared to alter gossypol levels in the plant. Because cadinene synthase belongs to a large multigene family comprising 16 members, there is the possibility that the individual member used for the transformation constructs, is not the ideal member for the blockage of constitutive gossypol production. Work is continuing as part of project CSP105C, to identity other members that may be of more use for the constitutive blockage of cadinene synthase expression. On the whole this project achieved its objectives with considerable success by cloning and characterising genes of possible importance in gossypol biosynthesis and using these genes for the production of transgenic cotton plants.

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?

Gossypol is toxic to monogastric animals and discolours cottonseed oil. Gossypol also lowers the nutritional value of cottonseed meal by binding to lysine residues. At present, cottonseed must be highly processed post-harvest in order to remove the gossypol before consumption. By generating gossypol-free cottonseed it is predicted that the expensive post-harvest processing would be unnecessary and the cotton seed products can be sold directly to the consumer. This would be expected to improve the gross worth of the cotton crop by increasing the value of cottonseed as a by-product.

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

There is currently no Intellectual property position covering this work. The seed specific promoter was obtained from an external laboratory and the use of this commercially would most likely require permission. There is however new seed specific promoters derived from cotton that are being characterised at this moment. These were discovered by Qing Liu and should be the intellectual property of CSIRO Plant Industry. In future we may use the new cotton promoters instead of the soybean promoters for cotton transformation. There is no patent claim to the rights of use of cadinene synthase for the manipulation of gossypol biosynthesis.

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

A method to detect gossypol levels by HPLC was developed by Chris Benson at the University of Western Sydney, under the supervision of Dr Gary Fitt. This method has been adapted for use at CSIRO Plant Industry in Canberra.

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

This field is growing in popularity and several international groups, particularly from the USA, are researching genes involved in terpenoid biosynthesis in cotton with the aim of generating gossypol-free cottonseed. To maintain a competitive stance in this field we have the advantage of a reliable and stable transformation system in cotton developed by the team of Dr Danny Llewellyn.

A list of publications arising from the research project

Belinda Townsend & Danny Llewellyn (1996) 'Molecular Biology of Gossypol Biosynthesis in Cotton' In: *Challenging the Future – Proceedings of the Eighth Australian Cotton Conference* (ed. G. A. Constable & N. W. Forrester), Brisbane, Australia, February 14-17, 1996, pp. 607-610

Belinda Townsend & Danny Llewellyn (1999) '(+)-δ-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, February 8-14, 1999, pp. 54

Belinda Townsend & Danny Llewellyn (1999) 'Molecular Biology of Gossypol Biosynthesis' Poster Presentation At: *Interactions and Intersections in Plant Signaling Pathways – Keystone Symposia on Molecular and Cellular Biology*, Coeur d'Alene, USA, February 8-14, 1999.

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.

The Molecular Biology of Gossypol Biosynthesis in Cotton

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Gossypol is a product of secondary metabolism unique to *Gossypium* species, including cultivated cotton. Gossypol is important to the plant for protection against pests and diseases. Unfortunately high levels of gossypol in the seed diminish the value of cottonseed because it must be removed from the oil and meal, as it is toxic to humans and non-ruminant animals. Ideally, the cotton plant should possess high gossypol levels in the plant whilst maintaining low levels in the seed. This characteristic has proven difficult to develop by traditional cotton breeding methods. We are now turning to molecular biology and genetic engineering of cotton to introduce this desirable trait.

Two genes of interest were targeted for the study of gossypol biosynthesis at the molecular level. The first gene encoded a cotton Cytochrome P450 enzyme, that was initially thought to be involved in the gossypol pathway induced by disease. However, detailed studies of where this gene was expressed suggested it was not involved in gossypol biosynthesis, but presumably some other biochemical pathway . Plants contain many P450 enzymes so we are continuing to search for the ones involved in gossypol biosynthesis.

The second gene cloned encoded (+)-δ-cadinene synthase, the first committed step in gossypol biosynthesis and a key regulatory branch point in the pathway. Cadinene synthase is a member of a large multigene family in cotton, each of which might be controlled in different ways to determine the distribution of secondary chemicals throughout the plant. The expression of the particular cloned gene was studied extensively and was shown to be strongly turned on in plants infected with the bacterial blight pathogen, more so in plants containing a resistance gene for this disease. The cadinene synthase protein was expressed in bacteria to allow antibodies to be made in rabbits for studies on where the protein is expressed in plants and to use for enzyme studies to determine the particular chemical reaction carried out by the cloned gene.

The cadinene synthase gene was also inserted into three gene constructs for the transformation of cotton. A seed specific promoter from the Soybean lectin gene was used to drive either the antisense cadinene synthase gene, designed to switch off gossypol production in the seed only, or the GUS reporter gene, designed to test the activity of this promoter in cotton. Analysis of GUS activity in the Lectin-GUS transformed plants demonstrated that this promoter was active early in seed development, before gossypol accumulation, and continued to be active at high levels throughout seed development, indicating it would be useful for controlling gene expression levels in the cottonseed. Preliminary analysis of the Lectin-antisense cadinene synthase plants, however, suggests that gossypol levels have not been reduced in these transgenic plants. A third construct was designed for the reduction of gossypol levels throughout the plant using the CaMV 35S with cadinene synthase in the antisense orientation. Early analysis of these plants also indicated no reduction in gossypol levels, however the cadinene synthase transcript and protein levels were significantly altered in comparison to the untransformed control plants when they were infected with bacterial blight. This result indicated that the particular cadinene synthase clone we have used may be important for the production of other secondary chemicals, related to gossypol, that are important for tolerance to bacterial diseases rather than to the gossypol produced in the seed. We have now cloned many different members of the cadinene synthase gene family and begun to characterise their expression patterns in cotton. This information will allow us to select a more likely candidate gene to continue this research as part of a new CRDC project CSP105C.