H. 2000.

January, August & Final Reports

Part 1 - Summary Project Details **REPORTS**

Please use your TAB key to complete parts1,2,4 & 5

CRDC Project Number: **CSP73C**

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

Project Title: Natural production of indigo in cotton fibres by genetic engineering

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Part 3 – Final Report Format

The points below are to be used as a guideline when completing your final report.

1. Outline the background to the project.

Colored Cottons Over the last few years there has been a resurgence in interest in naturally coloured cotton fibres to service a small, but growing, niche market in organic or natural textile products. These natural fibres can bring a premium to the growers over the conventionally produced white cotton because textiles and clothing produced from this cotton is sold at a high price to a small section of the community willing to foster products with minimal environmental impacts relative to the standard cotton production system with its heavy reliance on chemical pesticides and artificial fertilizers.

Growing coloured cotton began thousands of years ago, around 2700 B.C., in Indo-Pakistan, Egypt, and Peru. It was available in a very narrow range of colours - mocha, tan, gray and red-brown. The coloured variants were natural mutations in cotton collected by indigenous peoples when cotton was domesticated. Coloured types of cotton nearly disappeared with the Industrial revolution, kept alive only by craft hand-spinners, until it was revived in 1982 by Sally Fox, the founder of Foxfiber, who set about improving the naturally occurring brown and green coloured cottons using conventional plant breeding to improve the fibre properties and allow it be spun by machine. The final result was FoxFiber®, a long-fibred coloured cotton, which can be machine woven into cloth. The fabric created from FoxFiber® naturally coloured ranging from rich mocha to willow green. An expanded range of colours would be desirable to develop further this newly emerging market for naturally coloured fibres.

Conventionally produced white cotton has a somewhat tarnished image because of the heavy dependence on agricultural chemicals and this is being addressed to some extent by the introduction of genetically modified varieties (INGARD cotton) that are more tolerant to the common insect pests of cotton. The adoption of INGARD cotton has reduced chemical pesticide usage by 50% on about 180,000 ha of the 550,000 ha of cotton planted in Australia. However, agricultural production of the cotton fibre is not the only environmentally damaging aspect of cotton textile production, and downstream processing and dying can also lead to considerable pollution. A significant proportion of the world's cotton is dyed blue to produce the ubiquitous denim found in jeans and other fashion items. The dye for denim production is a synthetic form of indigo, a blue pigment once produced by the fermentation of indigo precursors produced from the woad plant. If cotton could be engineered to produce naturally blue coloured cotton fibres, there would be a significant commercial outcome through the capture of some of the international denim market and create a unique opportunity for the Australian cotton industry to establish a dominant position among World producers of cotton. This project has attempted to develop a number of systems for the production of indigo or other blue pigments in plants by using naturally occurring bacterial genes for blue pigment production.

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

- Demonstrate the production of indigo or indigoidine in a transgenic plant.
- Transform cotton with fibre promoter driving indigo production genes.

Unfortunately, we were never able to achieve the production of indigo in a transgenic plant. There have been reports that the Calgene company (now Monsanto) have produced indigo in cotton fibres, but when Dr Dave Stalker from Calgene visited the Division a couple of years ago and showed us pictures of his plants it was clear that the level of pigment expression was extremely low and since then Monsanto appears to have discontinued the project altogether. Calgene used a very similar approach to us, in expressing a naphthalene oxygenase enzyme from *Nocardia* sp. (known to make indigo in *E. coli*) and tryptophanase enzyme, which together should generate indole from tryptophan and convert this to indoxyl that can dimerise to indigo. Unfortunately indole is very unstable and normally sequestered within the active site of the anthranilate synthase enzyme that makes tryptophan. Removing tryptophan, an important amino acid from the available pool in a cell, to make indigo is always going to be difficult and none of the approaches we tried of targeting the enzymes to chloroplasts and trying to elevate the indole pool appeared to work.

The most promising strategy was to try to produce the different blue pigment, indigoidine, in plants as this appears to be synthesized from a different amino acid than indigo and might be more amenable to biochemical manipulation. We appear to have had more success with this approach and have isolated and characterised several genes responsible for indigoidine synthesis in the bacterium Vogsella indigofera and have shown that the key gene encodes a novel peptide synthetase that appears to be critical for pigment production in bacteria. Three genes were identified that were inferred to be necessary for us to make indigoidine pigments in plants and the appropriate gene constructs developed. We were not successful in introducing these genes into either cotton or tobacco, but using a seed transformation route we were able to express them in *Arabidopsis*. Many of the plants generated appeared to be pigmented (see below), but had poor vigour and most died. Those we could encourage to make seed did not appear to transmit the genes as the production of the pigment seems to be toxic to the plants. We have now generated transgenic Arabidopsis with each of the genes or combinations of the genes separately and will cross them together to see if we can produce pigment (particularly with one construct that has the peptide synthetase linked to a promoter that should only be expressed in petals, where it may not matter if the pigment is toxic). If we can better define how and where to express these genes to produce pigment we can then go back and attempt expression in cotton.

3. How has your research addressed the Corporations three outputs: Sustainability of natural resources, profitability and competitiveness, and/or people and communities?

Had we been able to generate indigo or indigoidine in cotton fibres we could have generated novel transgenic cotton germplasm that would have significant intellectual property and give Australian producers a unique product that would make them more competitive in the International market-place.

4. Detail the methodology and justify the methodology used.

Bacterial Pigment Genes for Indigo Production

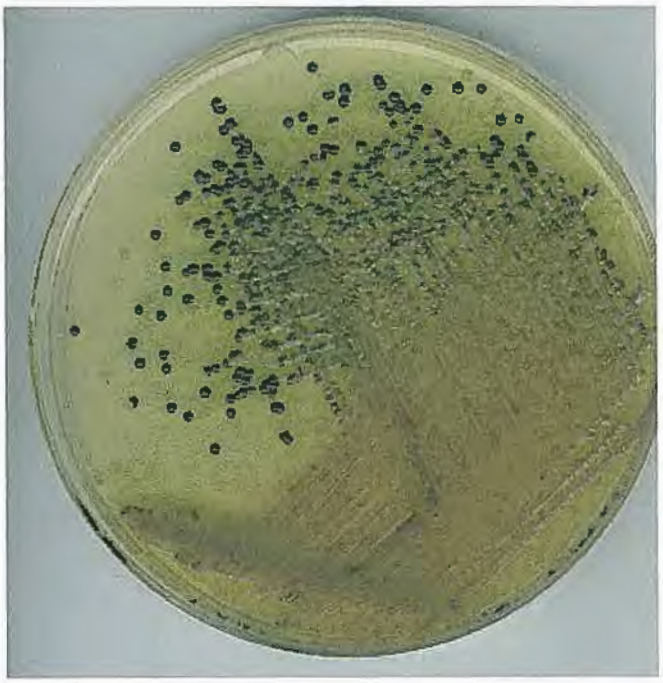
Plants already possess an array of vividly coloured pigments that determine the colour of both reproductive and vegetative organs, but the palette of colours is often tightly restricted within particular species or genera. Blue pigmentation, in particular, is confined to a small number of plant species and is generally, but not always, due to the water soluble anthocyanin pigment delphinidin. The engineering of blue pigmentation using plant genes has been attempted by the Australian Florigene Company, but success has been only partial, with the production of pinkish-mauve carnations such as "Moonshadow" or "Moondust", now sold commercially. A difficulty with this approach has been that the colour of the anthocyanin pigments is dependent on the presence of co-pigments and on the pH of the cell vacuole where they are stored. Alternative blue pigments with different chemistries would be desirable and pigmented micro-organisms should be a rich source of the genetic and biosynthetic capacity for blue pigment production, that could eventually be engineered into transgenic plants, and we have adopted the approach of trying to discover and express a number of different microbial genes in plants to make blue pigments.

Non-anthocycanin blue pigments with a range of colour shades are made by a number of bacteria, for example, pyocyanine is a light blue pigment made by *Pseudomonas aeruginosa* that might be useful for modifying flower colours. The textile industry, however, requires a darker blue pigment, like indigo.

Synthetic indigo is the commonly used dye to colour denim, and was historically derived from the fermentation of indoxyl glycosides made by certain plants, such as woad (*Isatis tinctoria*). It can also be produced in genetically modified bacteria using enzymes with broad substrate specificity, such as xylene oxygenase from *Pseudomonas putida* and naphthalene oxygenase from *Rhodococcus* Spp. which convert indole to the unstable precursor of indigo, indoxyl. Indoxyl spontaneously dimerises to indigo in the presence of oxygen. We attempted to express in plants a number of genes that can produce indigo in the bacterium *E. coli*, but had little success, presumably because of complex feedback controls and poor availability of the substrates for indigo production in plants.

Indigoidine - a Novel Blue Pigment System

An alternative strategy was investigated beginning in 1998 based on a completely different bacterium, *Vogesella indigofera*, and a different biochemical system for generating a blue pigment, called indigoidine. Indigoidine has very similar spectral properties to indigo including the coppery sheen seen with indigo at high concentrations and should provide an acceptable substitute for indigo if it could be produced in cotton fibres. Based on its chemical structure, however, it was clear that indigoidine was not derived from indole or tryptophan but possibly from glutamine, another common amino acid in plants and bacteria. The genes responsible for pigment production were identified by Dr Frank Van De Loo using a mutation strategy that knocked out pigment production in the bacteria following the random insertion of a mobile genetic element or transposon. Collating all the different mutants he was able to show that at least five different genes were involved in the actual production of the pigment (ie encoded the enzymes which make indigoidine) and a number more were involved in the control of when the pigment was produced. By successively moving the idigoidine genes into another bacterium, *E. coli*, he was able to show that only two of the five or six genes characterised appeared to be necessary for blue pigment production. So it might be possible that a relatively simple addition of just these two genes (*igiA* and *igiD*) might be all that was needed to make the pigment in plants. A third gene *igiB* may also be necessary for pigment production in plants as *E.coli* already has a similar gene that may carry out its function, but it was unknown whether plants have a similar enzymatic activity. The main focus of the project was therefore to express these genes in the model plant Arabidopsis to see if we could achieve the production of any pigment.



Promoters For Expression in Cotton Fibres

In another component of the project we have been working with Dr Sharon Orford and Prof. Jeremy Timmis at the University of Adelaide to test some of the potential fibre-specific promoters they had isolated from cotton. Sharon linked these to a GUS reporter gene and we have transformed them into cotton and have begun looking at the expression of the genes in both fibre and other tissues. All three of the initial promoters tested looked to be highly expressed in cotton fibres, although there appears to be some leakiness in the expression as we have found GUS activity in other tissues. Two of the promoters should be useful to target the expression of the idigoidine genes to predominantly developing cotton fibres and other floral tissues. Five more promoters are still being put into cotton to test their fibre-specific expression, as it will be desirable to have fibre-specific promoters expressed at different times during fibre development. It might be better, for example to express the indigoidine genes later in fibre development in case the pigment reduces fibre elongation or wall thickening. We do not have all the data yet to decide which of the promoters from Adelaide will be the most useful for expressing pigment genes in cotton fibres, but it is clear that a number of them could

be used at least just to test the possibility of targeting indigoidine expression there, if we are confident that we can achieve controlled expression in the model plant *Arabidopsis*.

5. Detail results including the statistical analysis of results.

In the first two years of the project we initially attempted two ways to make indigo in plants from indole, one of the precursors of tryptophan a common amino acid made in bacteria and plants. This used two different types of bacterial gene systems for the enzymes orfD from a purple-pigmented bacterium (*Chromobacterium violaceum*) and the second a two gene system (XylM and XylA) derived from a different bacterium (*Pseudomonas putida*) that degraded the organic chemical xylene. Both OrfD and xylM plus xylA expressed in transgenic tobacco or Arabidopsis (or cotton for OrfD), when driven by a strong constitutive promoter like 35S, did not produce any blue pigmentation, even when co-expressed with a tryptophanase gene derived from *E. coli* that should have generated a lot of indole, the substrate for the indigo pigment.

Isolation and Characterisation of Indigoidine biosynthetic genes

Genes involved in the production of the blue pigment, indigoidine, were identified by mutagenesis of *Vogesella indigofera* with transposon Tn*5*. Eight mutants were analysed in detail and shown to affect the structural-gene locus as well as three regulatory loci. Seven open reading frames were identified by sequencing a 10.2 kb genomic fragment at the structural locus, and five of these, *igiABCDE*, were ascribed putative functions in indigoidine biosynthesis and transport. Only *igiA* and *igiD* were required for intense blue pigmentation of *Escherichia coli*, and this pigment had a visible absorption spectrum (single major peak at ca. 612 nm) identical to indigoidine extracted from *V. indigofera*. The predicted IgiD protein had homology to non-ribosomal peptide synthetases, while IgiA had homology to 4'-phosphopantetheine transferase involved in making the peptide synthetase holoenzyme. By analogy with the biosynthetic pathway to nicotine blue, a chemically-similar pigment produced by *Arthrobacter oxidans*, a biosynthetic pathway is proposed from glutamine to indigoidine. A 2.9 kb genomic fragment was sequenced at one of the regulatory loci and contained homologs of the *Erwinia chrysanthemi* PecS/PecM regulatory couple. Production of indigoidine from *igiABCDE* cloned in *E. coli* required either mutation of *igiC*, or regulation by PecM, suggesting that transcriptional repression of *igiC* is the role of the *pecSM* locus in *V. indigofera*. Tn*5*-flanking sequences at the second regulatory locus identified homologs of sensor kinase and response regulator genes typical of classic two-component regulatory systems, while no homologies were detected with flanking sequence from the third regulatory locus. The structural genes for indigoidine biosynthesis provide the basic starting blocks for generating gene constructs for expression in plants.

Indigoidine Production in Transgenic Plants

Before he left CSIRO Dr van De Loo generated a construct using strong constitutive promoters such as the 35S promoter and sub-clover stunt virus promoters to drive the expression in plants of three of the idigoidine biosynthesis genes ((*igiA*, *igiD* *igiB*) and we attempted to use these for transformation of a variety of plants such as tobacco, cotton and Arabidopsis, initially with little success. In both tobacco and cotton, very little callus was generated, and it appeared that the construct was toxic to the tissues. In Arabidopsis, we were able to use a non-tissue culture based system for gene introduction, and this time we were able to get the indigoidine genes incorporated and expressed. The Arabidopsis system involves vacuum infiltrating the gene construct in the gene transfer bacterium *Agrobacterium* into the developing flower buds of an Arabidopsis plant and letting it continue to mature and produce seeds, some of which may become transformed with the genes. The seeds were then plated out on media containing the antibiotic kanamycin which allowed only plants containing the corresponding antibiotic resistance gene (that was on the same segment of DNA as the idigoidine constructs) to continue to grow after the seeds germinated. Of the few percent of plants that continued to grow several were an intense blue, almost purple colour (see pictureabove). Unfortunately most of the blue plants didn't develop any further, and those that did go on to flower seemed to lose their colour in the floral bolt, suggesting

that the genes were expressed, but that the pigment production was toxic when present throughout the plant. Screening of seed in the next generation confirmed that they were not resistant to kanamycin and that the pigment genes were not passed on.

A number of questions remain about the expression of the indigoidine genes in plants. Firstly, are all three genes necessary for pigment production or can some be dispensed with? The *igiD* gene is similar to peptide synthetase genes involved in the synthesis of cyclic peptide antibiotics in other bacteria and we expect that it is involved in the cyclisation of the amino acid glutamine or a derivative of it to form the basic structural unit of indigoidine. *IgiD* is therefore likely to be the critical enzyme necessary for indigoidine production. Peptide synthetases normally have an accessory protein that is needed for their correct function and this is likely to be *igiA*. These accessory proteins are not known in plants, so it is likely that *igiA* will also be essential. *IgiB* is a hydroxylase of unknown function, although similar genes are present in the genome of *E. coli* and it is not essential for indigoidine production in this bacterium, but we are unsure whether it is really necessary in transgenic plants. We have therefore generated new constructs with different combinations of the three genes and are testing them in Arabidopsis.

Second, the indigoidine construct, although driven by two different plant promoters is still quite active in bacteria and the Agrobacterium strain containing the gene constructs is itself intensely pigmented. This may reduce the efficiency of the transformation, but also complicates the assessment of pigment production in transformed plant tissues if any bacteria remain. We therefore modified the *igiD* coding region by incorporating a plant intron sequence. Introns occur in most plant genes and interrupt the coding part of the gene, but are removed before translation of messenger RNA into protein. Introns are not recognised in bacteria so this prevented expression of the *igiD* gene in the Agrobacterium, but it should be produced normally once transferred into the plant where the introns are spliced out during expression of the gene.

Finally, although we were able to express indigoidine production genes in plant, the plants were severely affected and many failed to develop beyond a few leaves. More precisely regulated expression of the genes to a particular plant organ or tissue might give less toxic side-effects and allow clearer assessment and inheritance of the pigment genes. We modified the promoter driving the igiD gene as this is the main biosynthetic enzyme and have left the other genes expressed everywhere throughout the plant. The petals of the flower are not essential for growth and reproduction so this seems like an ideal tissue in which to express indigoidine synthesis and would provide a useful demonstration of the potential of the genes to be used for floriculture. We have therefore coupled the expression of the *igiD* gene (containing an intron) to a petal-specific promoter rather than the ubiquitously expressed 35S promoter used previously. We have used the snapdragon chalcone synthase promoter used by Florigene to express the delphinidum pigment gene in the "Moonshadow" transgenic carnation as this has been shown to be petal-specific.

Transforamtions of Arabidopsis have been carried out with the 35S *igiD* or CHS *igiD* constructs as well as a 35S *igiA*, 35S*igiA* + S4*igiB*, and 35S*igiA*+35S*igiD*. In all cases the *igiD* gene contained an intron and as expected this prevented pigment production in the *Agrobacterium*. Unfortunately, we were only able to obtain a few stunted, pigmented plants with the *igiA+igiD* construct, indicating that only these two genes are necessary, but none of these survived transfer to soil, so incorporation of the intron has perhaps made them more efficiently expressed in plants and hence more toxic than the original intron-less construct. We have yet to complete the analysis of the transgenic plants with the other constructs and will need to cross them together before we would expect pigment to be produced. Of particular interest will be the 35S*igiA* and CHS*igiD* lines to see if pigment can be generated in petals.

Fibre-specific promoters The work on fibre-specific promoters has identified a couple of cotton promoters (promoters from lipid transfer protein (ltp) or ltp-like genes) that give strong expression in fibres, but also in some other floral tissues. We are continuing to analyse the expression patterns of transgenic cotton lines expressing these promoter-GUS fusions and will complete the first set of transgenic lines over the next six months. Unfortunately, other promoters and constructs developed by Dr Timmis's group, that we are also introducing into cotton are not yet available for analysis and we have found it difficult to give this material a high priority when the staff working on this material are

committed to more commercially oriented projects with the CSIRO transgenic cotton breeding program. Judy Radik has been involved full-time just completing the experiments we had already initiated and is now working on a different project funded by one of our Industry partners. We are taking a lot of the experiments through to producing transgenic plants (as part of our core biotechnology project), but this material will have to be transferred to Dr Timmis and they will have to complete the analysis themselves.

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

We have clearly yet to generate the blue pigment in cotton fibres, but we have been able to demonstrate the production of blue pigment in a model transgenic plant, although they were very sick. It has became clear that more research is needed in getting these pigments expressed in an easily transformed model species before we could move into a difficult crop like cotton. This is obviously a very difficult project technically, as plants do not like to, or are incapable of making large amounts of indigo, or perhaps even indigoidine, without very sophisticated regulation of the partitioning of either the enzyme activities needed or the substrates used in the synthesis of these pigments. Simply expressing these pigment genes in the plants like we do for a Bt-toxin gene or a herbicide tolerance gene is insufficient to generate transgenic blue plants that can survive. It will be necessary to precisely regulate one or more of the genes in a very tissue specific way to overcome any problems with toxicity to the plant. New constructs and transgenic plants have been produced that should address some of these issues, but these still need to be analysed in detail. If over the coming year if we can demonstrate the regulated expression of the blue pigment, it should provide us with a reasonable basis to move forward and try to generate a blue coloured cotton variety that could be incorporated into our core cotton biotech program and we will need to request additional support at that stage from the CRDC or other Industry partners to achieve a proof-of-concept in cotton.

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.

The production of blue coloured cotton fibres is still a relatively high risk project as we still don't have a good understanding of how to control the production of potentially toxic pigments within a plant cell. More research is needed in an easily transformed model species before we again tackle the production of these types of pigments in cotton fibres.

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

CSIRO is till considering the possibility of taking out patents on the use of indigoidine genes to produce blue pigments in plants, but it will be important to have demonstrated controlled expression of the pigment in a tissue like flower petals, before we are confident of moving ahead with the technology.

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

Nothing significant to report.

10. State the recommendations on the activities or other steps that may be taken to further develop, disseminate, or to exploit the project technology.

The project is still not complete, but we have some promising indications that blue pigment production may be possible in a plant, but the toxicity of the products issue means that we still need to explore ways of expressing it without harming the plant cell. A number of transgenic Arabidopsis lines with the genes expressed separately has been produced, but these still need to be analysed and crossed together to see if we can control pigment production in a tissue specific manner and we will continue this with internal CSIRO resources until we can get the project to a stage where we can seek further

funding. At such a stage we will again approach CRDC to take the project into a proof-of-concept in cotton.

11. List the publications arising from the research project.

A manuscript entitled: Structural and regulatory genes controlling indigoidine production in Vogesella indigofera: Involvement of a peptide synthetase homolog. By F. Van de Loo, P. Keese and D. Llewellyn has been prepared for the Journal of Bacteriology and has been peer reviewed in the Division and is awaiting clarification of the Intellectual Property position before submission to the Journal.

Part 4 – Final Report Plain English Summary

You must submit a half to one page Plain English Summary of your research proposal that is not commercial in confidence, and that can be published on the World Wide Web. An electronic copy of the Plain English Summary must also be forwarded by e-mail (angela@crdc.org.au).

Naturally blue coloured cotton fibres would have value to the industry because they would alleviate the additional cost and environmental pollution caused when white fibres are dyed with indigo pigments to produce denim. Although some plants already make blue pigments in flowers, these are water soluble and highly dependent on the presence of other co-pigments and environment within cells and would not be suitable as dyes for cotton fibres. Bacteria however, make a range of blue pigments, including the indigo that is used to dye denim. This project attempted to isolate different genes from coloured bacteria and express them in plants to produce the blue pigments indigo and indigoidine.

Unfortunately we were never able to get indigo produced in plants despite trying several different strategies using different genes that could make the pigments in the common bacterium *E. coli* and targeting them to different compartments in the cell where the chemicals needed to generate the pigment are located.

More success was obtained in producing the pigment indigoidine that has the same colour as indigo, but a very different chemical structure. The genes for indigoidine production were previously unknown so we first had to clone and characterise them from the blue bacterium *Vogsella indigofera.* There were some very interesting features about the enzymes that make the blue pigment as they were similar to enzymes that normally make cyclic peptide structures with antibiotic activity produced by some bacteria and it was possible that the blue bacterium made indigoidine as an antibiotic compound or by-product of antibiotic production.

Once we had identified the 2-3 genes we thought necessary for the production of the blue pigment we developed gene constructs to express them in plants. We tried a number of plants including cotton, tobacco and Arabidopsis (a small weedy plant often used as an experimental model in modern plant science) but were only ever able to produce transgenic Arabidopsis plants containing these genes (as we later discovered, the blue pigment seems to be toxic to plants during tissue culture). The Aribidopsis plants were intensely pigmented – but very sick and many died or produced seed that had lost the genes and were no longer pigmented. New gene constructs have been developed to express each of the different indigoidine production genes independently, and particularly in Arabidopsis flower petals where it may not matter if the pigment produced is toxic, but we have yet to complete the experiment where the different genes are brought together by crossing to see if we can make the blue pigment in a controlled manner so that it can be passed on to its progeny.

Should we, over the next six months or so, be able to produce the pigment in Arabidospsis petals and make blue flowers we would be in a better position to approach the CRDC or Industry partners to translate this into a proof-of-concept in transgenic cotton plants and fibres.