FINAL REPORT

Part 1 - Summary Details

CRDC Project Number: **UA13**

Project Title: Evaluation of transgenic cotton with altered fibre traits

Project Commencement Date: 1/7/04 **Project Completion Date:** 30/6/06

Research Program: On Farm

Part 2 – Contact Details

Administrator: Chelsea DuBois, Assistant Grants Officer
Organisation: The University of Adelaide
Postal Address: Adelaide Research and Innovation
The University of Adelaide, Adelaide SA 5005
Ph: (08)8303 3716 **Fax:** (08)8303 3700 **E-mail:** chelsea.dubois@adelaide.edu.au

Principal Researcher: Dr Sharon Orford
Organisation: The University of Adelaide
Postal Address: Discipline of Genetics
School of Molecular and Biomedical Science
The University of Adelaide, Adelaide SA 5005
Ph: (08)8303 7540 **Fax:** (08)8303 4362 **E-mail:** sharon.orford@adelaide.edu.au

Supervisor: Prof Jeremy Timmis
Organisation: The University of Adelaide
Postal Address: Discipline of Genetics
School of Molecular and Biomedical Science
The University of Adelaide, Adelaide SA 5005
Ph: (08)8303 3013 **Fax:** (08)8303 4362 **E-mail:** jeremy.timmis@adelaide.edu.au

Signature of Research Provider Representative: _____

Part 3 – Final Report Guide (due 31 October 2006)

Background

Using biotechnology to confer useful agronomic and fibre traits will lower the cost and time required for producing improved cotton varieties and will promote environmentally-friendly farm practices. The benefits of genetic engineering of cotton in Australia have been realised in the form of Bt transgenic cotton and Roundup®Ready varieties but, despite the obvious potential, these techniques have not yet featured significantly in the improvement of fibre quality.

Genetic engineering of cotton fibre morphology requires both useful genes and appropriate expression of the genes in cotton fibres. Previous CRDC-funded research in our laboratory has aimed to address both these requisites, concentrating on genes which are expressed in fibres but not in other cotton tissues, such as leaves, stems and roots. This research resulted in the identification of an expansin gene which is expressed exclusively in cotton fibres. Expansins are interesting proteins which are thought to control plant cell growth by chemical modification of cell wall components, and could therefore play a critical role in determination of fibre quality and yield.

Secondly, we have identified six different control regions (promoters) within the cotton genome which directly control the fibre-specificity and timing of expression of genes. Fibre-specific promoters allow the expression of any particular transgene to be targeted to the fibres only, avoiding any detrimental effects of expression on growth and morphology elsewhere within the plant. Each of the six promoters was fused to a reporter gene, GUS, and, in transient assays, shown to direct reporter gene expression that was confined to the fibres. An important test of a biotechnological tool is its behaviour *in planta*, so the six promoter::GUS constructs were also used to transform whole cotton plants, from which a total of 47 transformed lines were recovered. Of these, T2 seed had been collected from 7 lines. Only one line was obtained for our strongest and most promising promoter, pPR6.

We have also used our promoters as tools to disrupt normal production of expansin in the cotton fibre. Four genetic constructs were made, in which the expansin gene is under the control of a different fibre-specific promoter. The constructs were designed to overexpress, underexpress or prolong expansin gene expression during fibre development, and 33 different transgenic cotton lines were recovered. Of these, T2 seed had been collected from 11 lines.

Objectives

Broadly, the objectives of the project were to determine the effects of perturbation of expansin gene expression on cotton fibre development and to evaluate six different gene promoters for their ability to drive gene expression in a fibre-specific manner. Specifically, the aims were to:

a. Obtain and catalogue transgenic cotton seeds from all experiments
b. Grow as many transgenic lines as possible to T2 and where appropriate, carry out quantitative GUS assays (for the promoter::GUS lines) or evaluate the fibres for changes in morphology (for the expansin misexpression lines).

To a large extent these aims were achieved for the specific experiments studied.

The progress of the work was significantly enhanced by the sourcing of glasshouse facilities at the Waite Research Precinct, Adelaide. This meant that large numbers of cotton plants could be grown together in the glasshouse as well as in our small number of plant growth

cabinets. We also initiated a collaboration with Dr Greg Constable (ACRI) in order to evaluate the fibre quality of the transgenic plants in which expansin is being misexpressed.

In addition to the above research, Sharon Orford and Jeremy Timmis co-supervised two PhD students, John Humphries and Damien Lightfoot, to the end of their CRDC scholarships, and two Honours students, Joanna Sundstrom (2005) and Henry Martin-Harris (2006).

Methods

This project aimed to analyse the gene expression and/or fibre characteristics of ten different transgenic cotton experiments, six in which a fibre-specific gene promoter is controlling expression of a reporter gene, and four in which the expansin gene is being misexpressed in cotton fibre cells. Due to position effects (differences in gene expression which result from the location of the transgene in the genomic DNA) it is necessary to analyse 4-5 independent lines from each transformation experiment. Ideally, homozygous or pure-breeding plants should be studied. The methodology proposed was as follows:

1. Grow 4-8 T1 seeds of each transformed plant line and prepare genomic DNA from a small sample of seedling leaf tissue.
2. Test each plant for the presence of the transgene using the polymerase chain reaction (PCR) and sequence-specific primers. Discard negative plants.
3. Conduct Southern analysis on genomic DNA of positive plants in order to determine the copy number of the transgene and to confirm different genomic contexts between independent lines.
4. Grow positive plants to maturity and collect T2 seed.

The above steps were to be carried out for every transformant line which is obtained. In addition, further transgenic seeds sent from Canberra were to be manually ginned, catalogued and included in the project. The steps below were to be carried out for (at least) our most promising fibre-specific promoter, pPR6, and all of the expansin misexpression experiments.

For the promoter::GUS experiment(s):

5. Plant T2 seed of positive transformant lines and test at the seedling stage as described in steps 1, 2 and 3 above.
6. Conduct preliminary GUS staining of various cotton tissues from T2 plants to check for reporter gene expression in the fibres.
7. Collect tissues from positive plants, including fibres at various timepoints, and conduct quantitative GUS assays on protein extracts in order to determine the strength and timing of each promoter.

For the expansin misexpression experiments:

5. Plant T2 seed and test at the seedling stage as described in steps 1, 2 and 3 above.
6. Obtain pure-breeding lines for 4-5 lines per experiment by testing 12-15 progeny for presence of the transgene; if they are all positive then the line is homozygous for the transgene
7. Send pure-breeding lines to ACRI for measurement of fibre characteristics (HVI)
8. Determine levels of expansin gene expression in various tissues of homozygous plants by real time PCRs in comparison to wild-type levels.

Results

The final transgenic cotton seeds were obtained in August 2005 and the manual ginning and cataloguing completed. T1 seeds from a total of 43 lines were therefore recovered for the expansin misexpression experiment (four constructs) and from a total of 54 lines for the GUS reporter experiments (six constructs). Results from the two separate experiments are considered in detail below:

Promoter::GUS transgenic cotton

Only two of the six experiments were continued in this grant, namely pPR6 (our strongest and most promising fibre-specific promoter) and the expansin promoter. There were two reasons for our new focus on the expansin promoter as well as pPR6; firstly, that it tied in with our experiments on the gene itself, and secondly, that the expansin promoter had been used in experiments by Drs Yuan and Llewellyn at CSIRO PI in Canberra. All promoter::GUS experiments are summarised in the table below, with the two experiments of particular interest highlighted in yellow.

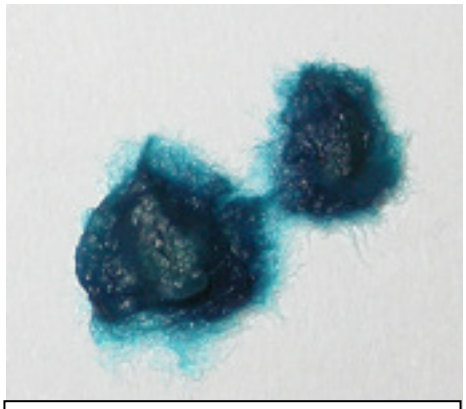
TABLE 1: Summary of transgenics with promoter::GUS reporter fusions

Construct name	Composition	Construct sent to Canberra	Expt	First seeds obtained	No. lines recovered	T2 seed collected (no. lines)
p3GUS1.5	pFS3 promoter::GUS	June 1999	T312, T313	Feb 2002	6 (6)	4 (4)
p6GUS1.4	pFS6 promoter::GUS	June 1999	T311	Jan 2004	1 (1)	1(1)
p18GUS1.2	pFS18 promoter::GUS	June 1999	T310	Jan 2003	10 (9)	6(5)
p17GUS1.2	pFS17 promoter::GUS	Nov 1999	T332	July 2003	24 (7)	7 (7)
pGUSEX1.2	pFS14 promoter::GUS	June 1999	T315	Feb 2002	3 (3)	1 (1)
pGUSEX1.4	Expansin promoter::GUS	June 1999	T316	July 2003	10 (4)	-
				TOTAL	**54 (30)**	**19 (18)**

One transgenic line only was obtained for the pPR6 experiment. T1 seeds were planted and seed collected from positive plants, as determined by PCR. Tissue was collected from positive T2 plants and preliminary staining carried out to determine whether the reporter gene, GUS, was being expressed in the cotton fibres. Testing of five separate plants showed only slight GUS staining in the young fibres of one plant, and the result was confirmed by quantitative GUS assays (on fibre aged 6-30 DPA and other tissues), which showed slightly elevated fluorescence in 6 DPA fibres, but the result was not significant. The lack of GUS expression in our one transgenic line of pPR6 could be due to a number of factors which cause gene silencing, including position effects.

Three transgenic lines were obtained for the expansin promoter experiment, one of which had been grown to T2. T1 seed from the two remaining lines was planted and T2 seed collected from positive plants. Preliminary GUS staining of tissues from all three lines showed that two were expressing the GUS gene in young fibres (an example is shown in Figure 1).

FIGURE 1: Staining of expansin promoter transgenic lines for GUS activity



6 DPA cotton ovules from line T315-4, showing blue staining of the fibres, indicative of GUS expression

6 DPA cotton ovules from line T315-49, showing lack of blue staining in the fibres

Quantitative MUG assays (on fibre aged 6-30 DPA and other tissues) carried out on one line, T315-4, showed that the expansin promoter was driving GUS expression in a strongly fibre-specific manner, and that expression peaked during fibre elongation and decreased as the fibre matured (result not shown). This exciting result shows that the expansin promoter is working as expected and could be a useful tool in cotton transgenics.

Expansin misexpression transgenics

There are four different experiments in which expansin gene expression is being up-regulated, down-regulated, or prolonged during fibre development. A number of different lines were obtained for each, as summarised in the table below.

TABLE 2: Summary of transgenics with expansin misexpression constructs

Construct name	Composition	Expected expansin expression	Construct sent to Canberra	Expt	First seeds obtained	No. T1 lines recovered *	T2 seed collected (no. lines)*
pPR6EXP	pFS6 promoter::EXP	increased	Dec 1999	T338	Jan 2003	10 (7)	9 (6)
pFS18AEXP	FS18A promoter::EXP	prolonged	Dec 1999	T336	Jan 2003	10 (9)	5 (5)
pFS18BEXP	FS18B promoter::EXP	prolonged	Dec 1999	T337	Dec 2002	16 (14)	5 (5)
pPR6antiEXP	pFS6 promoter:: antisense EXP	decreased	Jan 2000	T334	Dec 2002	7 (4)	6 (3)
					TOTAL	**43 (34)**	**25 (19)**

Our aim was to send four independent lines from each experiment for testing of fibre quality in the HVI machine at ACRI, a total of 20 lines to be sent in two batches of 10. We tested a total of 28 lines for homozygousity by PCR, as outlined above, and identified 14 pure-breeding lines (Table 3).

TABLE 3: Summary of homozygousity testing

Construct name	Composition	Expected expansin expression	No. lines taken to T2	No. lines tested	Pure-breeding lines	Segregating lines	Lines sent to ACRI
pPR6EXP	pFS6 promoter::EXP	increased	9	8	3	5	3
pFS18AEXP	FS18A promoter::EXP	prolonged	5	5	2	3	-
pFS18BEXP	FS18B promoter::EXP	prolonged	5	8	4	4	2
pPR6antiEXP	pFS6 promoter:: antisense EXP	decreased	6	7	5	2	5

Seeds from 10 lines were sent to Dr Greg Constable at ACRI on 25th Jan, 2006 for assessment of fibre characteristics in comparison to untransformed cotton. Time restraints prevented testing and sending of further lines but homozygousity testing is ongoing as part of project UA17.
Genomic DNA was extracted from leaves of transgenic plants and Southern blots performed in an effort to determine the transgene copy number and discriminate between lines. However a number of technical difficulties were encountered and the results were inconclusive.

1. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

The project aimed to complete the analysis of our transgenic cotton plants, thereby capitalising on the results of previous CRDC-funded research and, for a moderate investment, rounding off a number of years of funding to our laboratory by the CRDC. The project is ongoing, with the results of fibre analysis of the expansin misexpression lines yet to be received. In addition, only one promoter::GUS transgenic line has been fully analysed. However, these results contribute significantly towards our ultimate aim, namely the production of cotton varieties with improved fibre characteristics.

2. Please describe any:-
   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.); and
   c) required changes to the Intellectual Property register.

The outcomes of this research could be commercially significant and may require protection. Our previous advice has been that the research results are too preliminary to act upon, and further work is required to clarify exactly what is the innovation and its benefits for industry.

Conclusion

3. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

Results from this work represent a significant step towards our broad biotechnology aims, namely to produce cotton varieties with improved fibre characteristics and to develop molecular biological tools for cotton fibre improvement. The research has two main components, namely:

1. Characterisation and evaluation of genes with significant roles in the determination of cotton fibre characteristics, and;
2. Assessment of a bank of cotton gene promoters for their ability to drive fibre-specific gene expression in transgenic cotton plants.

We are close to determining whether changing the expression of a fibre gene, expansin, will alter commercially significant fibre characteristics in a measurable way. In the longer term, we expect the project to provide novel, partially characterised germplasm for use in cotton breeding programs aimed at improving cotton fibre properties. In addition, we are testing, *in planta*, a valuable bank of molecular tools which would allow expression of any gene in a defined manner in cotton fibre cells. These resources will lower the cost and time required for producing cotton varieties with improved fibre characteristics, the commercial cultivation of which will ensure the profitability and international competitiveness of the Australian cotton industry.

Extension Opportunities

4. 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.

There is a clear path for this project in terms of future research and development of the project technology. Having completed the initial testing phase in 10 transgenic cotton lines, it only remains to measure the fibre properties and, in the event of an interesting result, relate any changes to altered expansin gene expression. In the case of testing of the fibre-specific promoters, reporter gene expression has to be quantified in several lines for each of the four experiments.

Results from the first component will contribute valuable information on the gene(s) involved in cotton fibre development, as well as providing novel germplasm for use in cotton breeding programs. The second component will provide a range of extensively characterised gene promoters which could be used to express any gene specifically in fibre cells and in a defined temporal manner. These promoters, after full evaluation, will be available for use in other cotton research, such as gene misexpression studies (like those carried out in this project) or in the production of coloured cotton fibres. We would look to the CRDC for direction as to how best to supply these resources to the cotton breeding and biotechnology community.

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

Conference presentations during the tenure of this project:

D Lightfoot, S Orford and J Timmis, ISOLATION AND CHARACTERISATION OF COTTON BOLL WALL-SPECIFIC PROMOTERS, Genetics Society of Australasia Conference, Auckland, New Zealand, June 2005 (poster)

J Humphries, S Orford and J Timmis, ANALYSIS OF GENES INVOLVED IN COTTON FIBRE INITIATION, Genetics Society of Australasia Conference, Auckland, New Zealand, June 2005 (paper)

Publications:

Orford, S.J., Delaney, S.K. and Timmis, J.N. (2006) Genetic modification of cotton. In Gordon, S. and Hsieh, Y.-L. (eds) "Cotton: Science and Technology", Woodhead Publishing Inc, *in press*

Orford, S.J., Malone, K.M., Lightfoot, D.J. and Timmis, J.N. (2006) Evidence for alternative splicing of MADS-box transcripts in developing cotton fibre cells of *Gossypium hirsutum* (upland cotton), *in preparation*

Part 4 – Final Report Executive Summary

The Australian cotton industry occupies a niche market in optimal fibre quality, but this must be constantly developed in order to "stay ahead". Biotechnology provides an opportunity to continually improve fibre quality at lower cost and time, and in a more targeted way, than conventional plant breeding. Genetic improvement of cotton fibre morphology requires both useful genes and appropriate expression of the genes in cotton fibres. Previous CRDC-funded research in our laboratory has aimed to address both these requisites, concentrating on genes which are expressed in fibres but not in other cotton tissues.

We have identified six different controlling regions, or promoters, within cotton DNA which directly control the fibre-specificity and timing of expression of genes. Fibre-specific promoters allow the expression of any particular transgene to be targeted to the fibres only, avoiding any detrimental effects of expression on growth and morphology elsewhere within the plant. Each of the six promoters was fused to a reporter gene, GUS, and used to transform whole cotton plants. A large number of transgenic lines were recovered. Quantitative GUS assays were carried out the tissues of one transgenic line, showing that the reporter gene was strongly expressed in fibres only and that expression peaked during the elongation phase of growth.

One gene which is only expressed in cotton fibres encodes an interesting protein called an expansin. Expansins are thought to control plant cell growth by chemically modifying components of the cell wall, chiefly cellulose. As cellulose comprises such a large percentage of the cotton fibre, it could be that expansin proteins play a critical role in determination of fibre quality parameters such as length. Four genetic constructs were made, in which the expansin gene was placed under the control of four different cotton promoters, designed to alter normal expansin expression. The gene constructs were used to transform whole cotton plants and a large number of transformed lines were recovered. Ten lines have been screened for homozygousity and sent to the ACRI where the effects of the transgene on fibre properties such as length, strength and micronaire will be tested.

Results from this research will contribute valuable information on the role of the expansin gene in cotton fibre development, as well as providing novel germplasm for use in cotton breeding programs. In addition, it will provide a valuable bank of molecular tools which would allow expression of any gene in a defined manner in cotton fibre cells. Such tools could be used in other research aimed at producing fibres with improved or innovative properties.