98/063 2002. H VPMS ANU4C

January, August & Final Reports

REPORTS

Part 1 - Summary Details

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

CRDC Project Number: ANU4C

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

Project Title: Cloning genes to manipulate cotton fibre cellulose production for improved fibre traits

Project Commencement Date: 06-Nov-98 **Project Completion Date:** 06-Feb-02

Research Program: Plant Breeding and Biotechnology

Part 2 – Contact Details

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Signature of Research Provider Representative:

Part 3 – January & August Report Format

(Maximum four pages)

1. **What were your major project objectives for the past year? (Please list).**

2. **Which of these objectives have been achieved?**

3. **Which objectives were not achieved and why? (Please provide detail of any problems you have had during the year).**

4. **What are your specific project objectives for the coming financial year?**

5. **What aspects of your research project do you envisage having problems with in the coming year and why?**

   NOTE: This question is aimed at identifying areas in which CRDC may be able to implement assistance to help avoid potential problems. ***Needs to also ask how they plan to rectify these problems

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

7. **To what extent have your research results to date been disseminated to other researchers growers or the industry?**

8. (a) **How will your research results be useful to other researchers /growers /industry in the next year?**

   (b) **How do you intend to communicate these results or findings?**

9. **Were there major highlights in your work over the last six months? Please give a brief outline.**

10. **Are changes to the Intellectual Property register required?**

You may also submit a separate confidential report of information, which should be included in the report but which you reasonably consider is confidential information.

Part 4 – Project Variations for January Report

Detail and justify any variations to the original project proposal that you anticipate for the coming financial year (July to June).
(Eg Variations to outcomes or objectives, project time-line, budgets, or personnel).

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.

Cellulose is a crystalline ß-1,4 glucan found in all higher plants and comprises over 90% of the dry matter of the mature cotton fibre. Selection for improved cotton fibre properties has not usually targeted cellulose characteristics directly but comparisons of fibres with different properties suggest that varietal differences between fibres often reflect unconscious selection for differences in cellulose properties. For example, changes in the quantities of cellulose produced, in the timing of production and in physical properties such as chain length all impact on fibre properties. For many years the genes involved in cellulose production in plants remained elusive.. However, our work identified the first gene involved in this process. It encoded the catalytic subunit of cellulose synthase, the enzyme that sequentially adds the glucose units onto the growing cellulose chain. This gene is currently protected by patent. The work exploited our collection of cellulose mutants to identify the gene and most importantly to prove its function and its ability to change the physical properties of cellulose. (The cellulose deficiency causes radial swelling of the roots, so the mutants are referred to as *rsw* mutants. An *Arabidopsis* gene can be used to identify equivalent genes in cotton by standard methods of molecular biology and then the *Arabidopsis* mutant can be used to rapidly prove the function of the cotton gene by showing that it restores wild type function to the mutant.

The basis of this project was to identify the genes which were affected in two of the other cellulose deficient mutants of the collection, *rsw2* and *rsw3*. Cellulose production, like most biosyntheses, is almost certain to be controlled by several genes whose products may, for example, activate or inhibit the already identified catalytic subunit in response to environmental or developmental changes or may be required in some other capacity for cellulose to be generated. It is the identification of those additional genes, their functional characterisation and their protection for use in Australian cotton breeding that was the basis for undertaking this project.

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

1. Cloning of the *Arabidopsis RSW2* gene. Completed.

2. Isolation of the cotton *RSW2* ortholog. Completed.

3. Complementation of the *Arabidopsis rsw2* mutant with the cotton *RSW2* ortholog. Completed.

4. Cloning of the *Arabidopsis RSW3* gene. Completed.

5. Isolation of the cotton *RSW3* ortholog. Completed.

6. Complementation of the *Arabidopsis rsw3* mutant with the cotton *RSW3* ortholog. Not completed.

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

Our work has focussed on profitability and competitiveness through identifying and, where possible protecting by patent, genes for cellulose production that may be useful in producing cotton varieties with improved fibre traits. The genes identified from the research project have the potential to be involved in transgenic approaches

to improve fibre qualities of cotton, or could be used in traditional breeding approaches, to determine if any desirable fibre qualities are closely linked to the identified genes.

4. Detail the methodology and justify the methodology used.

Standard techniques used in molecular biology were successfully employed during the project. These included map based cloning to isolate the *Arabidopsis RSW2* and *RSW3* genes, DNA sequencing and sequence alignments to identify and characterise both genes. The Ds transposon-tagging approach described in the initial application for identifying *RSW2* was abandoned following sequencing of the RSW2 interval on *Arabidopsis* chromosome 5. Fibre specific cotton cDNA libraries constructed by Dr Sharon Orford (Adelaide University) and Dr Yingru Wu (CSIRO, Division of Plant Industry) were screened for cotton orthologs of *RSW2* and *RSW3*. Standard plant transformation vectors and transformation protocols were used to complement the root swelling mutants with the respective cotton ortholog.

5. Detail results including the statistical analysis of results.

RSW2: The *Arabidopsis RSW2* gene was identified following publication of the genomic sequence for an extended region of the chromosome to which we had mapped the *rsw2* mutation. Two candidate genes in the region were identified and sequencing of one of these (*KOR*) identified a single nucleotide change from wild-type. Confirmation that *KOR* was responsible for the defect in cellulose synthesis in the *rsw2* mutant was achieved through complementation of *rsw2* with a genomic copy of the *KOR* gene. KOR encodes a membrane bound endo-1,4-ß-glucanase, which may be involved in cleaving the glucose building blocks from a lipid linked intermediate. These short glucan chains can then either be added onto the end of the extending cellulose chain or form the starting point for initiation of a new chain. The cotton ortholog of *RSW2/KOR* was identified from an 18 dpa fibre cDNA library from Siokra 1-4 (kindly provided by Dr Sharon Orford). PCR primers were designed from analysis of three Expressed Sequence Tags that were available in the database. These cDNA clones showed strong homology to Arabidopsis RSW2, and together spanned the entire coding region. PCR primers designed at the beginning (5' end) and end (3' end) of the gene were used to amplify a full length copy of the cotton *RSW2* ortholog. Sequencing of the cotton gene revealed that it was 82% identical at the amino acid level to *Arabidopsis RSW2*, and contained all of the important domains for function. An overexpression construct was generated containing the cotton *RSW2* cDNA under the control of the constitutive CaMV promoter 35S. The *Arabidopsis* mutant *rsw2* was transformed with this construct and the transgenic plants generated had roots that did not swell. However, although the root swelling phenotype of *rsw2* was complemented by the cotton *RSW2* gene, the root hairs were not, and remained swollen in all transformants. The wild-type parent was also transformed with the cotton overexpression construct. Roots and root hairs were wild-type in appearance. Careful growth measurements and cellulose determinations are now being carried out on the next generation of mutant and wild type plants to determine if the overexpressed gene generates any potentially useful traits.

RSW3: The Arabidopsis *RSW3* gene was identified by map based cloning. The mutant gene was linked to a marker on chromosome 5 at a distance of 0.27cM. This region of chromosome 5 had been sequenced as part of the *Arabidopsis* genome sequencing program, and further analysis suggested a number of candidate genes that could potentially play a role in cellulose synthesis. Five of these genes were

sequenced in the *rsw3* mutant. One contained a single base pair difference from wild-type. Confirmation that this gene was indeed *RSW3* was achieved through complementation of the *rsw3* mutant with a genomic copy of the gene. *RSW3* encodes the catalytic subunit of glucosidase II, an ER-resident enzyme involved in N-glycosylation of proteins, and in their proper folding on the way through the ER to their final destinations. One partial cotton EST was identified from the database which showed strong homology to the *Arabidopsis RSW3* gene. Primers designed to this clone, were used to amplify a product from Dr Sharon Orford's fibre cDNA library. This gene specific fragment was used to screen another cotton fibre library from Dr Yingru Wu. Two clones were identified from this screen but neither was full length. The degree of similarity between these genes and the Arabidopsis *RSW3* gene was very high. Screening of Dr Sharon Orford's cDNA library with various primers designed to the cotton ortholog was also unsuccessful.

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

The *Arabidopsis RSW2* and *RSW3* genes were cloned and sequenced, completing objectives 1 and 4 stated in section 2. *RSW2* encodes a membrane bound endo-1,4-ß-glucanase which is allelic to the previously identified *KOR* gene. Patenting of the *RSW2* gene was not pursued due to prior publication of the *KOR* gene sequence, and the prior publication of an abstract by a Japanese group disclosing the link between KOR and cellulose synthesis. *RSW3* encodes the catalytic sub-unit of glucosidase II. This enzyme plays an important role in N-glycosylation of proteins and is directly involved in the correct folding of these proteins in the quality control pathway. This gene (although it has a large effect on cellulose levels) will affect a large number of processes that involve glycoproteins. The fact that the *RSW3* gene is not directly involved in cellulose synthesis led to a decision not to patent it. Cotton orthologs of both *RSW2* and *RSW3* were identified (completing objectives 2 and 5 in section 2). The cotton *RSW2* ortholog was full length and showed high homology to the *Arabidopsis* gene. When expressed from the CaMV 35S promoter the gene was able to complement the *rsw2* mutant (objective 3), thus confirming its function in cellulose synthesis and that functional interchange between cotton and *Arabidopsis* was possible. The cotton *RSW3* ortholog was not full length (5′ third missing), although the available sequence showed strong homology to the *Arabidopsis* gene. Due to the indirect role that the gene product plays in controlling cellulose synthesis it was decided that further work on complementation (objective 6) would not be of any benefit to the ultimate aim of manipulating cellulose in cotton fibres. This objective was therefore not completed.

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 a result of our work, the cotton industry now has a significantly clearer picture of how plants produce cellulose, the dominant component of the cotton fibre. Cellulose synthesis requires the activity of two enzymes in the plasma membrane and the proper functioning of the N-glycosylation/quality assurance pathway that operates in the endoplasmic reticulum within the cytoplasm. Analysis of *rsw* mutants has been central to identifying all three requirements. The work will impact on the cotton industry in 3 ways:

1. Our demonstration that the cotton ortholog of *RSW2* functionally replaces the *Arabidopsis* gene directly demonstrates the high similarity of the cellulose synthesis pathways in the *Arabidopsis* model and the cotton fibre. This shows that *Arabidopsis* mutants can be used not only to identify novel genes but also to functionally characterise cotton genes when these have been identified. The strategy should have broader application.

2. The genes identified as encoding these enzymes provide possible targets for transgenic approaches to modify cellulose production and potentially fibre properties. Some of these are currently being pursued (see (2) under Future Research Needs).

3. The genes identified are strong candidates to underlie some of the loci affecting cotton fibre properties when these are studied in mapping programs.

Future Research Needs

1. Continued analysis of the *rsw* mutant collection remains a promising route to identify more novel genes required for cellulose synthesis. We recently identified in the existing mutant collection a further 10 lines that are cellulose deficient. On current indications, about half may represent new loci. Their characterisation will be pursued through an ARC Linkage Grant with Aventis. It is hoped work will begin later this year and the three year project will probably bring our mutational analysis of cellulose synthesis in *Arabidopsis* close to completion.

2. Applications of the genes in cotton. As noted elsewhere in this final report, we are continuing to investigate the effects on growth and cellulose production in *Arabidopsis* of overexpressing the cotton ortholog of *RSW2* identified by the CRDC project. Promising results would make cotton transformation experiments attractive to investigate fibre properties and we would seek collaboration with those having this technology and possibly further CRDC funding to pursue it.

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

The constructs generated during the course of the project will be available for testing in cotton. The sequences of the genes cloned during the project will be available for testing for linkage to loci involved in improved fibre qualities. A patent application may be submitted for the cotton *RSW2* gene depending on the results of our ongoing analyses of the transgenic *Arabidopsis* plants.

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

N/A

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

(a) **to further develop or to exploit the project technology.** Initial observations of the phenotypes of the *rsw2* mutant and wild-type containing the 35S::cotton RSW2 construct have encouraged us to undertake further analyses of the T2 generation. If the preliminary observations are born out, we will seek advice regarding the potential value and patentability of the technology.

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

The cloning of the *Arabidopsis RSW3* gene is currently being written up and it is anticipated that it will be submitted in the next month to The Plant Journal. Subject to patenting, we anticipate reporting our findings on the cloning of the cotton *RSW2* ortholog and its complementation of the *rsw2* mutant, to a scientific journal.

11. List the publications arising from the research project.

Lane DR, Wiedemeier A, Peng L, Höfte H, Vernhettes S, Desprez T, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Betzner AS, Williamson RE (2001) Temperature sensitive alleles of *RSW2* link the KORRIGAN endo-1,4-ß-glucanase to cellulose synthesis and cytokinesis in *Arabidopsis thaliana*. Plant Physiology 126, 278-288

Williamson RE, Burn JE, Hocart CH. Cellulose synthesis: mutational analysis and genomic perspectives using *Arabidopsis thaliana*. Cellular and Molecular Life Sciences 58, 1475-1490

Submitted

Williamson RE, Burn JE, Hocart CH. Cellulose synthesis: major steps towards the mechanism. Trends in Plant Science

In preparation

Burn JE, Hurley UA, Birch RJ, Arioli T, Cork AC, Williamson RE. The cellulose-deficient *Arabidopsis* mutant *rsw3* is defective in glucosidase II, an enzyme processing N-glycans during ER quality control.

Burn JE, Hurley UA, Birch R, Williamson RE. A cotton ortholog of the KORRIGAN endocellulase complements the *Arabidopsis rsw2-1* mutant (submission subject to intellectual property considerations).

12. Are changes to the Intellectual Property register required? No

Part 4 – Final Report Plain English Summary

Cellulose is the structural carbohydrate that makes up over 90% of the mature cotton fibre. Some varietal differences in fibre properties have been traced back to the different cellulose properties shown by those varieties. As gene technologies continue to develop, opportunities to intervene much more directly to influence cellulose production in the fibre emerge. To use these technologies to improve fibre traits, we must first know which genes to use and how to use them and second, we must have the freedom to use them commercially without cotton growers paying high licence fees to overseas companies owning the technologies. Our project goals were to identify genes involved in cellulose production and, where appropriate, to protect them for commercial use.

The mechanism by plants produce cellulose have only recently begun to emerge. We have collected mutants of the model plant *Arabidopsis* which make much less cellulose. We call them *radial swelling* or *rsw* mutants because of their swollen roots. By finding which one of the plant's 25000 genes is defective in each mutant, we can identify which genes are needed for cellulose production. Before approaching CRDC, we had proved the value of the approach by showing that the first of these mutants was defective in a gene which encodes an enzyme extending the growing cellulose chain. During the CRDC project, we identified two more enzymes required for cellulose synthesis. The first provides an enzyme which cuts cellulose chains into smaller fragments, at first sight a rather surprising part of any machinery for making cellulose. We think it may carry out a "cut and paste" reaction in the early stages of cellulose synthesis before the individual cellulose chains crystallise into microfibrils. The corresponding gene was also isolated from cotton and we know it performs the same function because it can correct the cellulose deficiency in the *Arabidopsis* mutant. Work is continuing on whether overexpressing this cotton gene has any beneficial effects on cellulose production. A second mutant was defective in an enzyme that processes the chains of sugars which are attached to many plant proteins. Without that processing, we think that some proteins required to make cellulose do not function properly so reducing cellulose production. A cotton version of the gene was also identified.

Our work has therefore identified two new genes required to produce the cellulose which gives cotton fibres so many of their important properties and experiments on their value in manipulation cellulose production continue.

Part 5 – January Supervisor Report (Scholarships Only)

The Scholarship Recipient's Supervisor is to provide a brief statement on the Recipient's progress and achievements during the relevant year and whether the Recipient is fulfilling the requirements of the postgraduate or undergraduate course in which the Recipient is enrolled.