

## Final Report: CRDC Project UA4C

### Characterisation of Cotton Fibre-specific Gene Promoters

11th June, 1996 - 31st March, 1997

#### 1. Background

The aim of this project was to capitalise on work completed during tenure of a CRDC/ APRA PhD Scholarship<sup>1</sup>. Five gene products (messenger [m]RNAs) were isolated which are expressed at high levels in Siokra 1-2 cotton fibres and low levels in other parts of the cotton plant. The promoter region from one of these genes, *FSltp1*, was isolated and sequenced, and it was proposed to assess the ability of this and other promoters to control tissue-specific expression of a GUS reporter gene in transgenic cotton plants.

The majority of this work focussed on the preparation of gene constructs using a GUS expression vector. A plasmid DNA vector was obtained from Dr Danny Llewellyn (CSIRO Division of Plant Industry, Canberra) which contains a GUS reporter gene, upstream of which is a "polycloning site" into which promoter DNA fragments could be inserted. Work in this area was hampered by the deficiency of suitable restriction sites for cloning into the GUS vector.

#### 2. Results

##### 2.1 Cotton LTP Gene Promoters

The most abundant fibre-specific mRNA (pFS6) encoded a lipid transfer protein (LTP) which is present in cotton as a gene family. Two genomic clones previously isolated, namely  $\lambda$ FS6(A) and  $\lambda$ FS6(B), were identical and contained two genes, *FSltp1* and *FSltp2*. Sequence analysis showed that both genes encode LTP-like proteins and do not exactly match the sequence of the fibre-specific cDNA, with *FSltp2* differing markedly.

Two DNA fragments located upstream of *FSltp1* were subcloned into the GUS vector. Previous experiments with cotton gene promoters<sup>2,3,4</sup> have shown that promoter sequence of 2.3 kb is sufficient to drive fibre-specific gene expression in whole transgenic cotton plants. The *FSltp1* DNA fragments, sized 3.5 kb and 4.4 kb, were therefore expected to contain the complete promoter for the *FSltp1* gene, without any coding sequences. A further GUS construct, which contains a 2.2 kb promoter region from *FSltp1*, was generated from the existing 3.5 kb construct by dropout.

A further thirteen genomic clones were isolated by screening a Siokra 1-4 cotton genomic library (obtained from Dr Danny Llewellyn) with the fibre-specific LTP cDNA. Twelve of the clones were identical to each other in their restriction patterns but differed from that of  $\lambda$ FS6(A) and  $\lambda$ FS6(B), whilst the remaining clone was different from all previously-isolated clones. Sequence analysis of subclones derived from a representative of the twelve similar clones, namely  $\lambda$ FS6(D), showed that it contained a gene which was identical to *FSltp2*. Subcloning and sequencing of the coding fragments from the remaining clone,  $\lambda$ FS6(C), showed that it contained both *FSltp1* and a third, distinct LTP gene, termed *FSltp3*. The sequence of *FSltp3* differed from the two LTP genes previously isolated and from the fibre-specific cDNA. Constructs were made by cloning two DNA fragments located upstream of *FSltp3* into the GUS vector.

##### 2.2 Sequencing and Characterisation of Other Genomic Clones

Another fibre-specific cDNA, pFS17, appeared to encode a member of a class of well-characterised proline-rich structural proteins, or PRPs. The cotton PRP is presumed to have a structural role in the cell

wall of the developing cotton fibre. Extensive screening of the Siokra 1-4 genomic library with pFS17 sequences failed to identify any corresponding genomic clones.

The nature of the protein encoded by a third fibre-specific clone, pFS18, was unknown. Two genomic clones containing pFS18-like sequences had been previously isolated and one clone,  $\lambda$ FS18(Q), had been partially characterised. Sequence analysis revealed minor sequence differences between the genomic clone and the full-length pFS18 cDNA. DNA was prepared from the second clone,  $\lambda$ FS18(O) and restriction analysis of the clone showed it to be different from that of  $\lambda$ FS18(Q). A 4.4 kb *SacI* fragment was subcloned into a plasmid vector but sequence analysis showed that the gene contained in  $\lambda$ FS18(O) was identical to the gene encoded by  $\lambda$ FS18(Q) and consequently also differed from the pFS18 cDNA sequence. Several GUS constructs were generated using promoter fragments from genomic clone  $\lambda$ FS18(Q).

All clones and GUS constructs are summarised in the table below.

<u>Construct</u>	<u>Size of inserted promoter fragment</u>	<u>Genomic clone of origin</u>	<u>Cotton variety</u>	<u>Gene</u>
pGUS6(B)4.4	4.4 kb	$\lambda$ FS6(B)	Siokra 1-2	<i>FS1tp1</i>
pGUS6(B)3.5	3.5 kb	$\lambda$ FS6(B)		<i>FS1tp1</i>
pGUS6(B)2.2*	2.2 kb	$\lambda$ FS6(B)		<i>FS1tp1</i>
pGUS6(C)2.5*	2.5 kb	$\lambda$ FS6(C)	Siokra 1-4	<i>FS1tp3</i>
pGUS6(C)1.8	1.8 kb	$\lambda$ FS6(C)		<i>FS1tp3</i>
pGUS18(Q)0.9	0.9 kb	$\lambda$ FS18(Q)	Siokra 1-2	pFS18-like
pGUS18(Q)1.0	1.0 kb	$\lambda$ FS18(Q)		pFS18-like
pGUS18(Q)3.1*	3.1 kb	$\lambda$ FS18(Q)		pFS18-like
pGUS18(Q)4.3	4.3 kb	$\lambda$ FS18(Q)		pFS18-like

### 2.3 Particle Bombardment of Cotton Tissues for Transient Assays of Promoter Sequences

Transient assays were used to assess the ability of the promoter fragments to control tissue-specific expression of the reporter gene. A pilot experiment was performed to test the amenability of cotton fibres to transformation by particle bombardment. Cotton tissues including petals, leaves, 6 DPA fibres and 12 DPA fibres together with tobacco leaves were bombarded in quadruplicate using the Helium particle accelerator (Dr Anna Koltunow, CSIRO Division of Horticulture, Adelaide). Gold particles were coated with the plasmid construct pNTG<sup>+</sup>, which contains the GUS gene linked to a constitutive promoter which should drive GUS expression in all cotton tissues. Assays for GUS activity were carried out in conjunction with a positive control to show that the bombardment of cotton tissues and transient GUS expression were largely successful.

Bombardment of cotton leaves produced many foci of localised GUS expression. Petal and tobacco leaf were also successful but bombardment of 12 DPA fibre was unsuccessful, most likely due to the small target area. However, bombardment of 6 DPA fibres produced many patches of GUS expression. Some of these were clearly shown to be "hits" on epidermal cells of the ovule but other blue-staining fibres were separate from any other stained areas and it is probable that the latter staining resulted from direct bombardment of the fibre cells themselves.

Promoter fragments from each of the three different genes (indicated by asterisks in the table above) were tested for their ability to control fibre-specific gene expression by particle bombardment of

leaf and fibre tissue. The positive control, used in the pilot experiment described previously, produced foci of GUS expression in both fibre and leaf tissue. However, each of the three constructs, namely pGUS6(B)2.2, pGUS6(C)2.5 and pGUS18(Q)3.1, generated GUS expression in fibre tissue *which was absent in leaf tissue*. This result indicates that the three genes are expressed in fibre tissue and that they are considered to be *fibre-specific*.

### 3. Future work

During Project UA4C, we obtained crucial evidence that three genes isolated from Siokra 1-2 and Siokra 1-4 are expressed specifically in cotton fibres. Future work will involve conformation of this result by further bombardments of leaf and fibre tissue. The constructs will then be made available to CSIRO Division of Plant Industry, Canberra to be tested for their expression patterns in whole plant transformants.

“Dropout” experiments could also be performed to make a range of gene constructs which contain different promoter fragments. Assays performed using the dropout GUS constructs would allow the region of interest to be confined to a smaller DNA fragment, which would contain all the sequences necessary for controlling fibre-specific gene expression. The region could then be analysed by sequencing.

### 4. References

- <sup>1</sup>Orford SJ (1996) Analysis of gene expression during cotton fibre development. PhD Dissertation, Adelaide, Australia
- <sup>2</sup>John ME (1995) Characterisation of a cotton (*Gossypium hirsutum* L.) fibre mRNA (Fb-B6). *Plant Physiol* **107**: 1477-1478
- <sup>3</sup>John ME, Crow LJ (1992) Gene expression in cotton (*Gossypium hirsutum* L.) fibre: cloning of the mRNAs. *Proc Natl Acad Sci USA* **89**: 5769-5773
- <sup>4</sup>Rinehart JA, Petersen MW, John ME (1996) Tissue-specific and developmental regulation of cotton gene FbL2A. *Plant Physiol* **112**: 1331-1341

