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Abundant mRNAs specific to the developing cotton fibre[1]

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Abbreviations: DPA, days post-anthesis; nt, nucleotides.

ABSTRACT

Five fibre-specific cDNA clones have been isolated by differential screening of a cDNA library from cotton fibres, a developmentally synchronous population of non-dividing cells. The genes corresponding to these cDNAs were expressed preferentially in fibre cells and exhibited differing patterns of temporal expression during fibre development. All of the cDNA clones correspond to abundant transcripts in fibre cells 13 days after anthesis. Sequence analysis revealed that two clones show homology to previously reported plant sequences, while the others may encode as yet uncharacterised protein products.

INTRODUCTION

The commercial cotton fibre is a product of Malvaceous plants of the genus *Gossypium*. Cotton fibres differentiate from single cells of the outer epidermis of ovules and originate at about the time of anthesis. The signals which trigger differentiation of an epidermal cell are unknown but "prefibre" cells can be discerned from other epidermal cells as early as 16 hours preanthesis (Ramsay and Berlin, 1976). The potential for fibre formation is present as early as nine days preanthesis (Davidonis, 1989). Fibre cells develop in four distinct but overlapping phases, beginning with an initiation period which is followed by extensive cell elongation (primary wall synthesis). Secondary wall deposition begins at 16 to 19 days post-anthesis (DPA) and continues until fibre maturity, the final phase in fibre development (Schubert et al., 1973; Basra and Malik, 1984). The degree of overlap between the phases of elongation and secondary wall thickening varies between cultivars and under differing environmental conditions (Benedict et al., 1973; Schubert et al., 1973; Jasdanwala et al., 1977). Overlap tends to be greater in long staple cultivars than in short staple cultivars (Naithani et al., 1981), and time of onset of secondary wall deposition may control final fibre length (Beasley, 1979). At 45 to 60 DPA, the fruit capsules (bolls) open and the 2 to 3 cm cylindrical fibre cells collapse to twisted, ribbon-like structures.

Cell wall biosynthesis is a major synthetic activity in fibre cells. The rate of cellulose synthesis increases rapidly at 16 DPA and cotton fibre cells are 95% cellulose at maturity (Young, 1986; Davidonis, 1989). Specific cell wall characteristics influence a number of textile properties in cotton. Of obvious importance are chemical composition of the wall, degree of cellulose polymerisation and amount of cellulose deposited (Rowland et al., 1976; Gupta et al., 1979; Timpa and Ramey, 1989). Fibre elongation is driven by turgor pressure generated by the influx and accumulation of potassium and malate in the enlarging central vacuole (Dhindsa, Beasley and Ting, 1975). The osmoregulation of these solutes may play an important role in the rate and

The economic importance of cultivated cotton has stimulated extensive studies of fibre and seed development. Manipulation during domestication has resulted in modern cotton varieties in which the fibre is many times longer and stronger than that of any wild ancestor. Fibre properties, particularly length, strength and fineness, require further improvement to meet economic pressures and to facilitate more efficient spinning technology (Colton, 1991). Because these characters are under genetic control (Basra and Malik, 1984), modification of fibre development through genetic manipulation could result in enhanced fibre quality parameters. Prior to this, an understanding of the regulatory elements and genes expressed in cotton fibres is required.

Since cotton fibres grow synchronously and represent single elongated cells (which are relatively homogeneous with respect to size), they are a suitable experimental system in which to study cellular and developmental events. The aim of this work was to isolate and characterise cDNA clones corresponding to mRNAs which are present in fibre cells but absent in other differentiated cotton tissues.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seeds of cotton, *Gossypium hirsutum* L., cv Siokra 1-2 (Cotton Seed Distributors, Narrabri NSW) were sown in soil and grown in growth cabinet conditions (day/night temperatures of 30°C/25°C, light/dark cycle of 16/8 hours). Fibres were manually harvested at various stages of development and the yield from each boll weighed and frozen in liquid nitrogen. Leaf and whole flowers were collected from mature plants and stored at -70°C. Seedling tissue, with the cotyledons and shoot apex removed, was harvested when the plants had reached a height of approximately 5 cm.

Differential screening of a cDNA library

Total RNAs were isolated from all cotton tissues using the method of Wan and Wilkins (1994) and the RNA stored in 80% ethanol at -70°C. Polyadenylated (poly(A)$^+$) RNA from leaf and 13 DPA fibre was affinity purified on oligo(dT)-cellulose columns (mRNA Purification Kit, Pharmacia, Uppsala, Sweden) according to manufacturer's instructions.

Double stranded cDNA was synthesised from 13 DPA fibre poly(A)$^+$ RNA using a commercial cDNA synthesis kit, according to manufacturer's instructions (Pharmacia, Uppsala, Sweden). cDNA was ligated into *Eco*RI-digested and dephosphorylated λZAP ®II vector (Stratagene GmbH, Heidelberg), packaged using *in vitro* packaging extracts (Promega, Madison, WI) and plated according to Stratagene instructions. Phage were lifted in duplicate onto Hybond-N$^+$ membranes (Amersham, UK) and differentially screened with ^{32}P-labelled single-stranded cDNA probes from poly(A)$^+$ RNAs of leaf and 13 DPA fibre.

To prepare single-stranded cDNA probes, 2 µg to 4 µg of poly(A)$^+$ RNA in 3 µl of DEPC-treated H_2O was heated to 65°C for 5 minutes and transferred to ice for 5 minutes. After briefly microfuging the sample, the following components were added in order: 1 µl DTT (10 mM), 20 U RNasin (Promega, Madison, WI), 3.1 µl Tris-HCl

(1.6 μg/μl, Amersham, UK), 1.3 μl each of dATP, dTTP and dCTP (10 mM), 120 μCi [α-^{32}P]dATP (Amersham, UK) and 1 μl MLV reverse transcriptase (200 U/μl, BRL, Gaithersburg, MD). The mix was incubated at 41°C for 2 hours.

Preliminary analysis of cDNA clones

Inserts from differentially hybridising λZAP®II plaques were rescued into the phagemid vector pBluescript®SK(-) (Stratagene GmbH, Heidelberg) for further manipulation. Plasmid DNA was purified using the alkaline lysis method of Sambrook et al. (1989). Polymerase chain reactions (PCR) across cDNA inserts was performed in 25 μl reaction mixtures containing 200 ng plasmid DNA, 1 x PCR reaction buffer (Bresatec, Adelaide), 0.4 mM each of dATP, dCTP, dGTP and dTTP, 4 mM $MgCl_2$, 0.7 μM each of T3 and T7 primers and 1.5 U of T*aq* DNA Polymerase (Bresatec, Adelaide). Initial template denaturation was at 94°C for 2 minutes followed by 25 PCR cycles of denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute and extension at 72°C for 1 minute.

Northern blotting, Southern blotting and hybridisation

DNA samples were digested with restriction endonucleases under conditions recommended by the manufacturer (Boehringer Mannheim, Germany). Following electrophoresis, DNA was transferred to a Hybond-N$^+$ membrane (Amersham, UK), following manufacturer's instructions. Tissue specificity of isolated cDNA fragments was assayed by Northern blotting. Approximately 10 μg samples of total RNA were size fractionated by electrophoresis on denaturing formamide agarose gels (Sambrook et al., 1989) and transferred onto Nytran®-plus (Schleicher and Schuell GmbH, Germany) following manufacturer's instructions.

Plasmid inserts requiring radioactive labelling were purified from an agarose gel using the freeze-squeeze method (Thuring et al., 1975). Probes were labelled with [α-^{32}P]dATP (Amersham, UK) using the GIGAprime DNA labelling kit (Bresatec, Adelaide).

Prehybridisation, hybridisation and washes of Southern blots, Northern blots and plaque lifts were carried out under conditions recommended by Amersham. Oligo(dA) (Amersham, UK) to a concentration of 5 µg/ml was added to hybridisation of cDNA library clones. Filters were exposed to X-ray film (Fuji RX) at -70°C in the presence of intensifying screens (DuPont Hi-Plus).

Sequencing of double-stranded DNA templates

Plasmid DNA for sequencing was purified by centrifugation through a Sepharose CL-6B chromatography column and sequenced using a Sequenase Version 2.0 kit (United States Biochemical Corp, Cleveland, Ohio) and [α-^{32}P]dATP (Amersham, UK). DNA sequencing reactions were electrophoresed in a 5 %(w/v) acrylamide, 7 M urea and 1 x TBE (0.9 M Tris-borate, 0.002 M EDTA) denaturing polyacrylamide gel using a BRL sequencing gel electrophoresis system. Sequence analysis was done using the GCG Sequence Analysis Software Package version 8 (Genetics Computer Group, Madison, WI, 1984). BLASTN and BLASTX were used to search nucleotide and protein sequence databases, respectively (Altschul et al., 1990; Gish et al., 1993).

RESULTS

A developing cotton fibre contains thousands of active genes (Graves and Stewart, 1988), among which will be those that are crucial to fibre differentiation. We have attempted to identify genes that are specifically expressed in 13 DPA fibres through a differential screening experiment. Screening of 2.3×10^4 recombinant complementary DNA (cDNA) clones with leaf and fibre mRNA probes resulted in the identification of 24 putative fibre-specific clones. Cross-hybridisation eliminated duplicate clones, reducing the putative fibre-specific clone population to six different sequence types (Table 1, columns 1 and 2). PCR across each insert revealed the relatively short length of the inserted cDNAs which ranged in size from approximately 0.2 to 2 kb.

To investigate the spatial and temporal expression patterns of the cDNA clones, RNA blot analysis was carried out using RNA from various cotton tissues and from fibres of various developmental stages. Five of the six clones (pFS3, pFS5, pFS17, pFS18 and pFS19) showed highly preferential hybridisation to fibre mRNA (Fig. 1), hybridising strongly to transcripts in fibre RNA but not to RNAs from leaf, whole flower or seedling tissue. One clone, pFS20, was unique in the clone collection and did not represent a fibre-specific transcript. Rather, pFS20 transcripts were present equally in fibre, leaf, flower and seedling tissues, producing constitutive signals comparable with those obtained for a cotton rDNA clone (not shown). This hybridisation pattern provides a useful positive control, reflecting RNA loading (Fig. 1a,b). On longer exposure, leaf, flower and seedling tissues showed weak hybridisation signals to each fibre-specific cDNA probe (not shown), indicating that transcription is not entirely restricted to the fibre or that products of genes with similar sequences cross hybridise to the cDNA probes. The temporal pattern of transcript accumulation varied between clones, as shown by hybridisation of cDNA inserts to RNA from fibres of varying ages (Fig. 1, lanes 6-20). Genes corresponding to pFS5, pFS19 and pFS17 appeared to be expressed early in fibre development, whilst the pFS3 gene is expressed uniformly in fibres aged 6 to 20 DPA and expression of pFS18 appeared to peak at 12 to 14 DPA.

The estimated sizes of the transcripts specified by the five fibre-specific clones ranged between 600 nt and 1500 nt as indicated (Fig. 1) and the pFS20 cDNA hybridised to a transcript of 300 nt. Based on a comparison of cDNA length and transcript size from RNA blots, it was concluded that full-length clones were not obtained for any of the fibre-specific mRNAs (Table 1, columns 4 and 5).

The relative abundance of fibre-specific transcripts was determined by using isolated cDNA inserts as probes to a larger sample of the 13 DPA fibre cDNA library consisting of 3.7×10^4 recombinants (Table 1, column 3). Fibre-specific transcripts make up a substantial proportion of the mRNA population in 13 DPA fibre tissue, ranging in these experiments from 0.16% (pFS3) to 0.65% (pFS19) of the mRNA.

Sequence information for cDNA clones corresponding to the five differentially-expressed mRNAs was used to determine potential functions of the encoded proteins. The longest single-insert clone in each group was chosen for sequence analysis (Table 1, column 1). The sequence of pFS5 shows 96% nucleotide identity with the corresponding region of a previously characterised fibre-specific cDNA, E6 (John and Crow, 1992). The 91 residue amino acid sequence derived from clone pFS5 is 91% similar to the 94 carboxy-terminal amino acids of the E6 protein (John and Crow, 1992). A significant difference is an in-frame deletion of nine nucleotides from pFS5 compared with E6, which results in the absence of three amino acids from the conceptual translation product of pFS5.

The sequence of pFS19 shows similarity to cDNAs encoding phospholipid transfer proteins (LTPs) a class of plant proteins thought to be involved in the biogenesis of cellular membranes (Arondel and Kader, 1990). The 58 residue amino acid sequence derived from clone pFS19 is homologous to LTPs found in spinach (Bernhard et al., 1991), tobacco (Fleming et al., 1992), maize (Tchang et al., 1988) and carrot (Sterk et al., 1991) and shares amino acid identities of 71%, 68%, 64% and 59%, respectively. In addition, the pFS19 nucleotide sequence is very similar (97%) to that of GH3, a cotton fibre-specific LTP cDNA recently isolated from *Gossypium hirsutum* L., cv. DES119 (Ma et al., 1995). The sequence of pFS19 shows an additional 17 bp

site for polyadenylation. The conceptual translation of the pFS19 cDNA shows 91% amino acid identity to the corresponding region of the GH3 protein, containing five amino acid substitutions of which only three are to amino acids with similar physical properties. Sequences of pFS3, pFS17 and pFS18 identified no definitive homologies in the nucleotide databases (GenBank and EMBL; September 1995).

DISCUSSION

The developing cotton fibre is a dynamic structure, the molecular profile of which is continually changing through development (Meinert and Delmer, 1977). Gross changes in protein composition occur in cotton fibres during the early stages of development, as seen by SDS-PAGE of *in vitro* translation products (Alexander, Williams and Delmer, 1983). Two-dimensional protein patterns of 10 DPA ovules and 10 DPA fibres revealed a few proteins which are not shared by the two tissues (Graves and Stewart, 1988). We have identified five fibre-specific mRNAs in fibre cells at 13 DPA, which is approximately the time of transition between the elongation and secondary wall deposition phases of fibre development (Basra and Malik, 1984).

The limitation of the differential screening technique is detection of abundant mRNAs which represent at least 0.1% of the total mRNA population (Sargeant, 1987). The abundance of each fibre-specific transcript in 13 DPA fibres has been estimated by probing the cDNA library with the cloned inserts. Values greater than 0.1% were obtained in all cases (Table 1, column 3). The apparent discrepancy between only 24 original clones and large numbers of hybridising plaques in the same cDNA library arose because only clearly identified plaques were picked. Many differentially hybridising plaques were overlooked in the original screen. The values in Table 1 are based on the assumption that each poly(A)$^+$ mRNA is equally clonable and that each clone contains a single cDNA insert. Multiple inserts were observed in a proportion of longer clones in this experiment, indicating that the mRNA abundance values may be an over-estimation. Relatively however, pFS19 transcripts, encoding the cotton LTP, are the most abundant fibre-specific mRNAs in 13 DPA fibres and comprise about 0.65% of the total mRNA population.

RNA gel blots confirmed the tissue-specificity of the majority of mRNAs that were detected by differential screening (Fig. 1). Transcripts corresponding to each of five cDNA sequences accumulated to high levels in 13 DPA fibre tissue but were present at low levels in leaf, whole flower and seedling tissue. While it is unknown

indicate a role for transcriptional control of gene expression in cotton fibre cells. Differential stability of mRNAs may also account for the distortions. Transcripts corresponding to pFS5, pFS19 and pFS17 increased in concentration as fibre development proceeded through primary wall synthesis (Fig. 1, lanes 6-16), and decreased at the onset of secondary wall synthesis. This may suggest a role for the corresponding proteins in primary wall synthesis or elongation of the cotton fibre. Transcripts of pFS3 were present during both the primary and secondary cell wall synthesis stages (Fig. 1a, lanes 6-20), suggestive of a continuous role throughout fibre cell development. The abundance of pFS18 mRNA appeared to peak at 12 to 14 DPA (Fig. 1b), the time of transition between the elongation (primary wall synthesis) and secondary wall deposition phases of fibre growth. It seems likely that the levels of at least some of the polypeptides involved in secondary wall synthesis may markedly increase during this transition. The protein(s) encoded by pFS18 may therefore be involved in secondary wall deposition.

Two of our five fibre-specific clones show substantial similarity to known proteins. The sequence of pFS5 showed similarity at the nucleic acid level and amino acid level to a previously characterised fibre-specific cDNA, E6 (John and Crow, 1992). The sequence differences are probably ascribable to allelic differences between *G. hirsutum* L., cv. Coker 312 and *G. hirsutum* L., cv. Siokra 1-2. The function of the E6 protein is unknown but it has been suggested to have a structural or enzymatic role in cotton fibre primary cell wall deposition. Predominance of pFS5 transcripts early in fibre development (Fig. 1a) contrasts with the expression pattern of E6, which showed maximum mRNA levels in fibres aged 15-22 DPA (John and Crow, 1992). However, early accumulation of this transcript correlates well with the immunodetection of the E6 protein product in Western analysis (John and Crow, 1992). Maximal E6 protein accumulation was observed to occur between 5 and 15 DPA and the discrepancy between this and the maximal presence of the corresponding transcript was attributed to differential stability of the protein. Environmental factors or varietal differences may be responsible for the observed difference in pFS5 and E6 expression patterns.

The pFS19 nucleotide sequence showed significant similarity to cDNAs encoding phospholipid transfer proteins (LTPs) from a variety of plant species. The pFS19 nucleotide sequence is very similar (97%) to that of GH3, a cotton fibre-specific LTP cDNA of *G. hirsutum* L., cv. DES119 (Ma et al., 1995). Again, these minor sequence differences may be attributable to allelic differences between the cultivars *G. hirsutum* L., cv. DES119 and *G. hirsutum* L., cv. Siokra 1-2. The temporal expression pattern of pFS19 (Fig. 1a) confirms the findings for GH3 (Ma et al., 1995). Plant nonspecific LTPs have several features in common including low molecular mass (9-11 kDa), high (basic) isoelectric point and an N-terminal signal peptide, characteristic of secreted proteins. Expression of LTP genes is under developmental and environmental control in higher plants, and expression is often tissue-specific (Bernhard et al., 1991; Sterk et al., 1991; Fleming et al., 1992). LTPs are generally encoded in plants by multigene families of at least two members, and different genes of the family may encode similar proteins that have distinct functions or expression patterns.

A number of different functions have been attributed to LTPs, including antibacterial and antifungal activity (Molina and García-Olmedo, 1993) and stress response (Torres-Schumann *et al.*, 1992). In several *in vitro* studies, it has been shown that nonspecific lipid transfer proteins can mediate the intermembrane transfer of phospholipids (for review see Kader, 1990). However, presence of the signal peptide is not easily reconciled with the presumed cytosolic function for LTPs in the transport of phospholipids. Carrot LTPs are proposed to be involved in the transport of cutin monomers to the outer epidermal wall, where they can be released and esterified in the growing cuticle (Sterk et al., 1991). LTPs may have a similar role in cotton fibres, which are coated by a waxy cuticle (Seagull, 1993), absent in other cotton cell types. The cuticle is deposited as the fibre elongates, consistent with predominance of pFS19 transcripts early in fibre development.

Screening of nucleotide databases was uninformative for three clones, pFS3, pFS17 and pFS18, probably due to incomplete sequence information. Rapid amplification of cDNA ends (5'-RACE) is being used to obtain the 5' ends of the fibre-

information derived from the full-length cDNAs may provide clues to possible functions of proteins encoded by fibre-specific mRNAs.

Knowledge gained from this work may allow manipulation of gene expression to modify cotton fibre characteristics such as length, strength and fineness. In addition, isolation of the corresponding regulatory sequences will allow accurate tissue targeting of heterologous gene expression specifically to the fibres in transgenic plants. Based on our analysis, the pFS19 gene is most active in cotton fibre cells, with much lower levels of transcripts present in leaf, flower and seedling tissue. The latter may be due to detection of transcripts derived from other members of the LTP gene family and this possibility is currently under investigation. In addition, we are using the pFS19 insert as a probe in the screening of a *G. hirsutum* L., cv. Siokra 1-2 genomic library to retrieve corresponding genomic clones. The fibre-specific promoter will be characterised by sequence analysis and by linkage to a GUS reporter gene system in transgenic plants.

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LEGEND TO FIGURE

Figure 1. Northern analysis of six putative fibre-specific cDNA clones.

(a) and (b) represent independant Northern blots with 10 μg of total RNA in each lane, sequentially hybridised with the specific probes indicated. Total RNA loadings in each track were monitored by hybridisation with cDNA clone pFS20 and shown in the lower panel of each blot. Approximate transcript sizes in nucleotides (nt) are indicated on the left. The pFS18 hybridisation pattern was obtained using a longer homologue, identified by rescreening the cDNA library with pFS18.

Table 1. Cotton cDNA clones with fibre-specific expression.

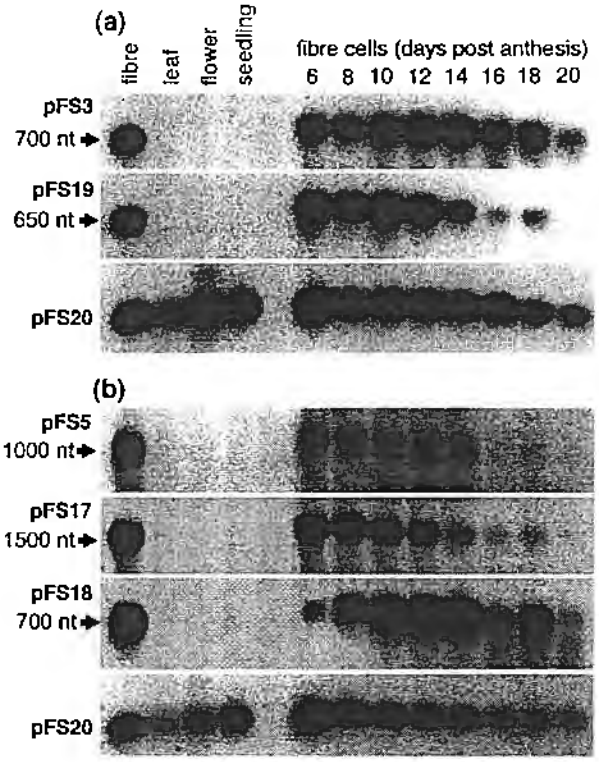
Representative cDNA clone	Number of clones in group	Transcript abundance in 13 DPA fibres (% of mRNA)[a]	Transcript size (nt)[b]	cDNA size (bp)[c]	Sequence homology[d]
pFS3	1	0.16	700	520	-
pFS5	6	0.47	1000	509	Fibre-specific cDNA clone E6 (John and Crow, 1992)
pFS17	3	0.23	1500	138	-
pFS18	1	0.18	700	98	-
pFS19	12	0.65	650	330	Plant phospholipid transfer proteins (Arondel and Kader, 1990)

[a] Estimated by probing a 13 DPA fibre cDNA library with the representative insert.

[b] Estimated by comparison with RNA molecular weight markers (Promega).

[c] Determined by sequencing entire cDNA insert. Value does not include poly(A)$^+$ tail.

[d] Using the BLASTN and BLASTX programs of the GCG software package, Madison, WI.


(a)
fibre
leaf
flower
seedling
fibre cells (days post anthesis)
6 8 10 12 14 16 18 20
pFS3
700 nt
pFS19
650 nt
pFS20
(b)
pFS5
1000 nt
pFS17
1500 nt
pFS18
700 nt
pFS20