RESEARCH ARTICLE

Isolation, characterization and mapping of genes differentially expressed during fibre development between *Gossypium hirsutum* and *G. barbadense* by cDNA-SRAP

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Abstract

Gossypium hirsutum and *G. barbadense* are two cultivated tetraploid cotton species with differences in fibre quality. The fibre of *G. barbadense* is longer, stronger and finer than that of *G. hirsutum*. To isolate genes expressed differently between the two species during fibre development, cDNA-SRAP (sequence-related amplified polymorphism) was applied. This technique was used to analyse genes at different stages of fibre development in *G. hirsutum* cv. Emian22 and *G. barbadense* acc. 3-79, the parents of our interspecific mapping population. A total of 4096 SRAP primer combinations were used to screen polymorphism between the DNA of the parents, and 275 highly polymorphic primers were picked out to analyse DNA and RNA from leaves and fibres at different developmental stages of the parents. A total of 168 DNA fragments were isolated from gels and sequenced: 54, 30, 38 and 41 from fibres of 5, 10, 15 and 20 days post-anthesis, respectively, and five from multi stages. To genetically map these sequences, 104 sequence-specific primers were developed and were used to screened polymorphism between the mapping parents. Finally, six markers were mapped on six chromosomes of our backbone interspecific genetic map. This work can give us a primary knowledge of differences in mechanism of fibre development between *G. hirsutum* and *G. barbadense*.

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Introduction

Cotton (*Gossypium* spp.) is one of the most economically important crops and the leading natural fibre crop in the world. The two cultivated tetraploid species, upland cotton (*G. hirsutum*) and extra-long staple cotton (*G. barbadense*), account for about 95% and 2% of the world's total production, respectively (National Cotton Council, http://www.cotton.org). Extra-long staple cotton has superior fibre quality but low yield, while upland cotton has high yield but low fibre quality. The contrasting and complementary traits of the two species make them widely used to dissect the genetic basis of yield and fibre quality of cotton (Mei *et al.* 2004; Lacape *et al.* 2005, 2010; He *et al.* 2007).

Cotton fibres are highly elongated single-cell trichomes that arise from the outer epidermis of the ovules. Fibre cell elongation occurs at a fast rate over a relatively long period and is uninterrupted by cell division. It involves four distinct but overlapping steps: fibre cell initiation, elongation, secondary wall biosynthesis, and maturation (Basra and Malik 1984; Ruan and Chourey 1998). Therefore, cotton fibres are an excellent experimental system for studying plant cell elongation and secondary cell wall synthesis (Kim and Triplett 2001).

There have been considerable work in cloning fibre-related genes by differential screening of fibre cDNA library (John and Crow 1992; John 1995; John and Keller 1995; Orford and Timmis 1998), cDNA differential display (DDRT-PCR) (Song and Allen 1997; Zhang *et al.* 2004), PCR amplification using gene-specific probes (Smart *et al.* 1998; Loguercio *et al.* 1999; Whittaker and Triplett 1999), and suppression subtractive hybridization (Liu *et al.* 2006; Wu *et al.* 2008). Such techniques are labour-intensive and time-consuming. Some have high levels of false positives, or are biased for high-abundance mRNA (Wu *et al.* 2008).

Recently, to explore the molecular mechanism of fibre development, large-scale gene discovery methods such as

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expressed sequence tag (EST) analysis (Arpat *et al.* 2004; Wu *et al.* 2005; Udall *et al.* 2006; Yang *et al.* 2006), microarray transcriptome analysis (Ji *et al.* 2003; Arpat *et al.* 2004; Lee *et al.* 2006, 2007; Shi *et al.* 2006; Wu *et al.* 2006, 2007; Gou *et al.* 2007; Taliercio and Boykin 2007; Alabady *et al.* 2008; Hovav *et al.* 2008), and, most recently, deep-sequencing technology have been used (Wang *et al.* 2010). The cotton species investigated included *G. arboream*, *G. raimondii*, *G. hirsutum* and *G. barbadense* (Udall *et al.* 2006; Tu *et al.* 2007). These studies gave an outline of cotton fibre development and helped us to identify candidate genes for further research. However, most of these studies focussed on one or different fibre development stages of one genotype, or comparing wild and mutant types. It is gratifying that some studies compared differently expressed genes between cotton species (Udall *et al.* 2007; Alabady *et al.* 2008; Chaudhary *et al.* 2008; Al-Ghazi *et al.* 2009; Hinchliffe *et al.* 2010; Rapp *et al.* 2010; Bao *et al.* 2011).

Beyond microarrays, cDNA-AFLP has also been used in cotton for transcriptional profiling (Ma *et al.* 2008), transcriptome mapping (Pan *et al.* 2007) and expression quantitative trait loci (eQTL) mapping (Liu *et al.* 2009, 2011; Claverie *et al.* 2012). Compared to cDNA-AFLP, cDNA-SRAP (sequence-related amplified polymorphism) is a cheaper and simpler tool for functional genomics and genetics. The method involves RNA extraction, cDNA synthesis, PCR amplification and electrophoresis. SRAP, first developed by Li and Quiros (2001), can amplify both DNA and cDNA. Li *et al.* (2003) constructed an F_2 transcriptome map based on *Brassica oleracea* cDNAs obtained from leaf tissue by cDNA-SRAP. The map consisted of 247 cDNA markers, and 169 marker sequences were similar to genes reported in *Arabidopsis*.

Because fibre quality is dramatically different between *G. hirsutum* and *G. barbadense*, identification of differences in genes and gene expression between them has the potential to provide useful tools for understanding the different mechanisms of fibre development, and it may also be possible to use them for improving cotton fibre quantity and quality through biotechnology. In this study, we used cDNA-SRAP to characterize differences in fibre development between *G. hirsutum* and *G. barbadense*, to isolate differently expressed genes between the two species, and to genetically map these genes to show their chromosomal distribution.

Materials and methods

RNA and DNA extraction

The plant materials used in this study were the mapping parents of the BC_1 mapping population, *G. hirsutum* cv Emian22 and *G. barbadense* acc. 3-79. Accession 3-79 has longer lint than Emian22 (figure 1a), while Emian22 has more fuzz than 3-79 (figure 1b). RNAs of developing fibres of 5 days post-anthesis (DPA), 10, 15 and 20 DPA were extracted from the parents by the method described by Zhu *et al.* (2005). DNAs and RNAs of young leaves were also extracted as controls; DNAs were extracted by the method of Paterson *et al.* (1993). DNAs and RNAs were checked by running them through 1.0% agarose gel.

cDNA synthesis and cDNA-SRAP analysis

The first strand cDNAs were synthesized using 3 μg RNA from each sample, following the instructions of Superscript® III RT kit (Invitrogen, Carlsbad, USA).

The SRAP primers (Em1–Em64 and Me1–Me64) for amplifying DNAs and RNAs of leaves and fibres were from Lin *et al.* (2009). PCR reaction, electrophoresis, and silver staining were performed as described by Lin *et al.* (2005).

Sequencing different bands and bioinformatics analysis

Bands that showed polymorphism between the two parents were cut from dried gels. DNA in each band was eluted with double-distilled water and reamplified for 34 cycles

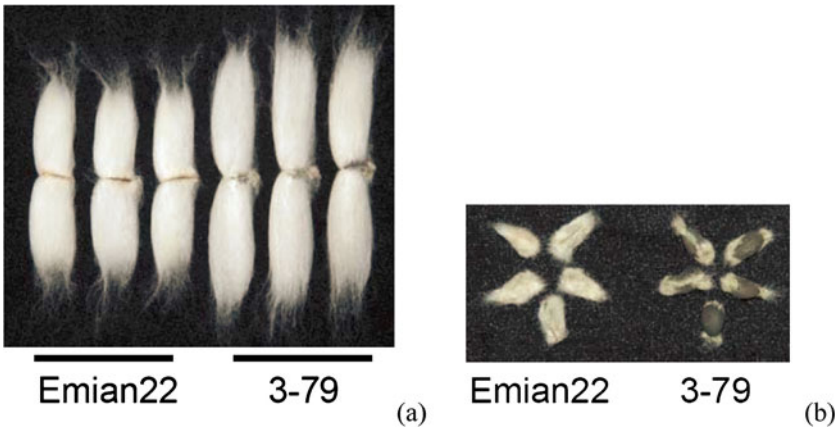

Figure 1. Differences in fibre lint (a) and fuzz (b) between Emian22 and 3-79.

as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR products from these bands were cloned; after checking on agarose gel, the positive clones were sequenced by BGI (Beijing Genomics Institute, Beijing, China). After removing the vector sequences and common sequences, these unique transcript-derived fragments (TDFs) were BLASTed against cotton ESTs with a threshold of E $\leq$ 1.0e-15 and the nonredunant protein database in NCBI using tBLASTx (http://www.ncbi.nlm.nih.gov) with a threshold of E $\leq$ 1.0e-5. Gene ontology (GO) was performed using BLAST2GO (Conesa *et al.* 2005; Götz *et al.* 2008) to functionally annotate these TDFs.

Marker development and genetic mapping

Primers were developed from the TDFs. The criteria for primer design were as follows: primer length 18–24 bp, optimum 20 bp; GC content 35–60%, optimum 50%; optimum annealing temperature 57°C; and PCR product size between 100 and 350 bp. Primers were named with a prefix CF plus numbers.

The primers were used to screen polymorphism between Emian22 and 3-79. PCR reaction mixtures (15 μL) consisted of 1× reaction buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.3 μmol/L primers, 30 ng template DNA, and 0.5 U *Taq* DNA polymerase (MBI). Amplification was carried out in a Bio-Rad thermal cycler with an initial 5 min at 94°C followed by 34 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C and lastly a 5 min final extension at 72°C. First, the amplified products were genotyped by electrophoresis using 6% denaturing polyacrylamide gels (acrylamide:bisacrylamide (19:1), 1× TBE) at room temperature and viewed by silver staining. Then the monomorphic primers were separated using 8% native polyacrylamide gels (29:1, acrylamide:bisacrylamide) at a constant 15 W at 4°C (single-strand conformation polymorphism technology, SSCP). After 3.5–4 h of electrophoresis the gels were observed by silver staining.

The polymorphic primers were used to genotype the whole BC_1 mapping population ((Emian22 × 3-79)×Emian22). Polymorphic loci were integrated into our interspecific BC_1 linkage map (Yu *et al.* 2011) using JoinMap3.0 (Stam 1993) with a logarithm of odds (LOD) threshold of 5.0 and a maximum recombination rate of 0.4. Map distances in centimorgan (cM) were calculated using the Kosambi (1994) mapping function.

Results

cDNA-SRAP differential display

The OD260/OD280 ratios of RNAs extracted from different tissues were 1.80 ~ 1.98, which indicated that the RNA quality was satisfactory for this study.

A total of 4096 SRAP primer combinations were used to amplify DNA of the mapping parents to select more polymorphic primer combinations for cDNA-SRAP differential display. A total of 275 polymorphic primer combinations were picked out to amplify DNAs and RNAs of different tissues. To ensure accuracy of the differential display results, every primer combination was repeated thrice. Compared to DNA amplification products, cDNA amplification products were fewer and polymorphism was lower. Upon electrophoresis, the amplification products of fibre cDNAs, leaf cDNAs and genomic DNAs were classified into (i) common bands, (ii) bands only from fibre and leaf cDNAs, and (iii) bands only from fibre or leaf cDNAs (figure 2).

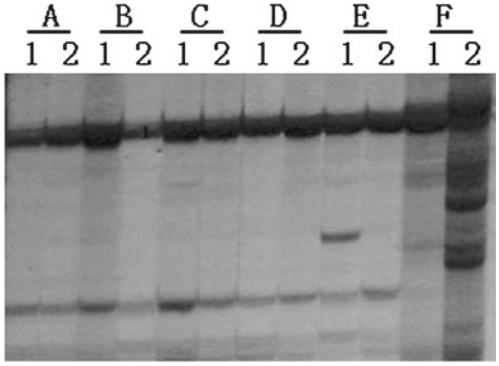

Figure 2. Amplification profile from different tissues produced by SRAP primer combination Me25Em26. (A) cDNAs from leaves; (B–E) cDNAs from fibres of 5 DPA, 10 DPA, 15 DPA and 20 DPA, respectively; (F) genomic DNA; 1, Emian22; 2, 3-79.

Isolation of cDNA-SRAP different bands

cDNA-SRAP bands of fibre cDNAs that were different between Emian22 and 3-79 were isolated and sequenced. A total of 168 clones were successfully sequenced and submitted to GenBank after removing the vector sequences (accession numbers: GT226793–GT226906 and GT228350–GT228403) (table 1; see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet). The lengths of these sequences were 200–800 bp, most of which were about 300 bp. The number of sequences from Emian22 was almost

Table 1. Distribution of special sequences at different fibre development stages.

Fibre development stage	Specific sequences from Emian22	Specific sequences from 3-79	From both parents	Total
5 DPA	38	16		54
10 DPA	16	14		30
15 DPA	20	18		38
20 DPA	35	6		41
Others (multi-stages)	4	0	1	5
Total	113	54	1	168

double that from 3-79. Table 1 also shows that the biggest difference between Emian22 and 3-79 was for 20 DPA fibre followed by 5 DPA fibre; the smallest difference was for 10 DPA fibre.

Annotation and functional classification of TDFs

The 168 TDFs were assembled into 110 unique sequences. In BLAST against cotton ESTs, only 14 sequences (12.73%) were found that matched cotton ESTs (table 2 in electronic supplementary material). Fifty-one sequences were found that matched proteins (table 3 in electronic supplementary material). GO analysis (level 2) showed that 23 sequences were mapped to 'cellular component', with 'cell part' and 'organelle' dominating this item; 34 sequences were mapped to 'molecular function', with 'catalytic activity' and 'binding' dominating this item; 29 sequences were mapped to 'biological process', with 'molecular process' and 'cellular process' dominating this item (figure 3).

Genetic mapping of TDFs

A total of 104 pairs of sequence-specific primers were designed based on the 110 unique sequences (table 4 in electronic supplementary material). These primers were used to screen polymorphism between the mapping parents by normal gel analysis and SSCP, and 61 primers produced clear bands and 15 primers showed polymorphism (CF03, CF07, CF13, CF22, CF28, CF44, CF54, CF65, CF67, CF77, CF85, CF86, CF93, CF97 and CF100). After genotyping the BC_1 population, seven polymorphic loci were obtained; among which five were dominant (CF03, CF44, CF93a, CF93b and CF100) and two codominant (CF77 and CF85) (dominant markers for Emian22 did not segregate in this BC_1 population).

Six of the seven polymorphic loci were integrated into our interspecific genetic linkage map, and were distributed on six chromosomes. Five loci mapped on chromosomes of At subgenome, and only one on Dt subgenome (figure 4).

Discussion

There have been many robust methods to isolate differentially expressed genes in plants. Among them, cDNA-SRAP is a general, easy-to-use and inexpensive method (Li *et al.* 2003). In this study, cDNA-SRAP was used to isolate genes expressed differently between *G. hirsutum* and *G. barbadense* during cotton fibre development. First, highly polymorphic primers were screened between parent DNAs, and then they were used to amplify fibre cDNAs with DNA and leaf cDNA as controls. Hundreds of polymorphic bands were identified. The results showed that polymorphic bands from Emian22 were significantly more than those from 3-79, which is in agreement with the observation that the period of fibre development in *G. hirsutum* is shorter than in *G. barbadense* (Chen *et al.* 2012).

cDNA-SRAP differential display revealed differences at 5 DPA between Emian22 and 3-79. Generally, cotton fibre lint initiates from −3 DPA to 0 DPA, and elongates from 3 DPA to 20 DPA; while fibre fuzz typically appears at 5 DPA (Lang 1938). Therefore, 5 DPA is the overlapping stage of fibre cell initiation and elongation, which determines the quality of fibre lint and fuzz. Emian22 generates more fibre lint and fuzz than 3-79 (figure 1b). It is reasonable that more polymorphic bands were isolated from Emian22. Another distinctive difference of gene expression between Emian22 and 3–79 was at 20 DPA. At this stage, fibre elongation basically stopped and secondary wall biosynthesis increased in *G. hirsutum*; while fibre elongation still continued to about 25 DPA in *G. barbadense* (Chen *et al.* 2012), which resulted in longer fibre lint in *G. barbadense* (figure 1a). So, it is understandable that the biggest difference of expression was observed at this stage. There were minor differences at 10–15 DPA, which is the fibre elongation stage in both Emian22 and 3-79, indicating

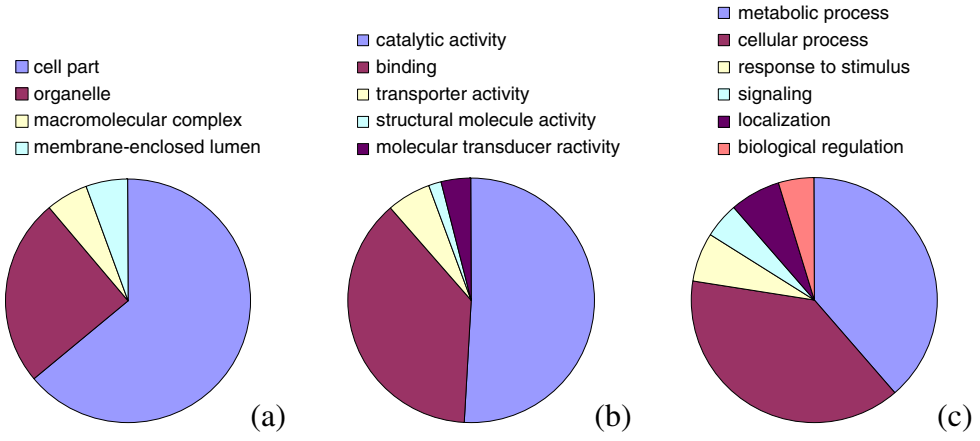

Figure 3. Functional classification of TDFs (transcript-derived fragments): (a) cellular component; (b) molecular function; (c) biological process.

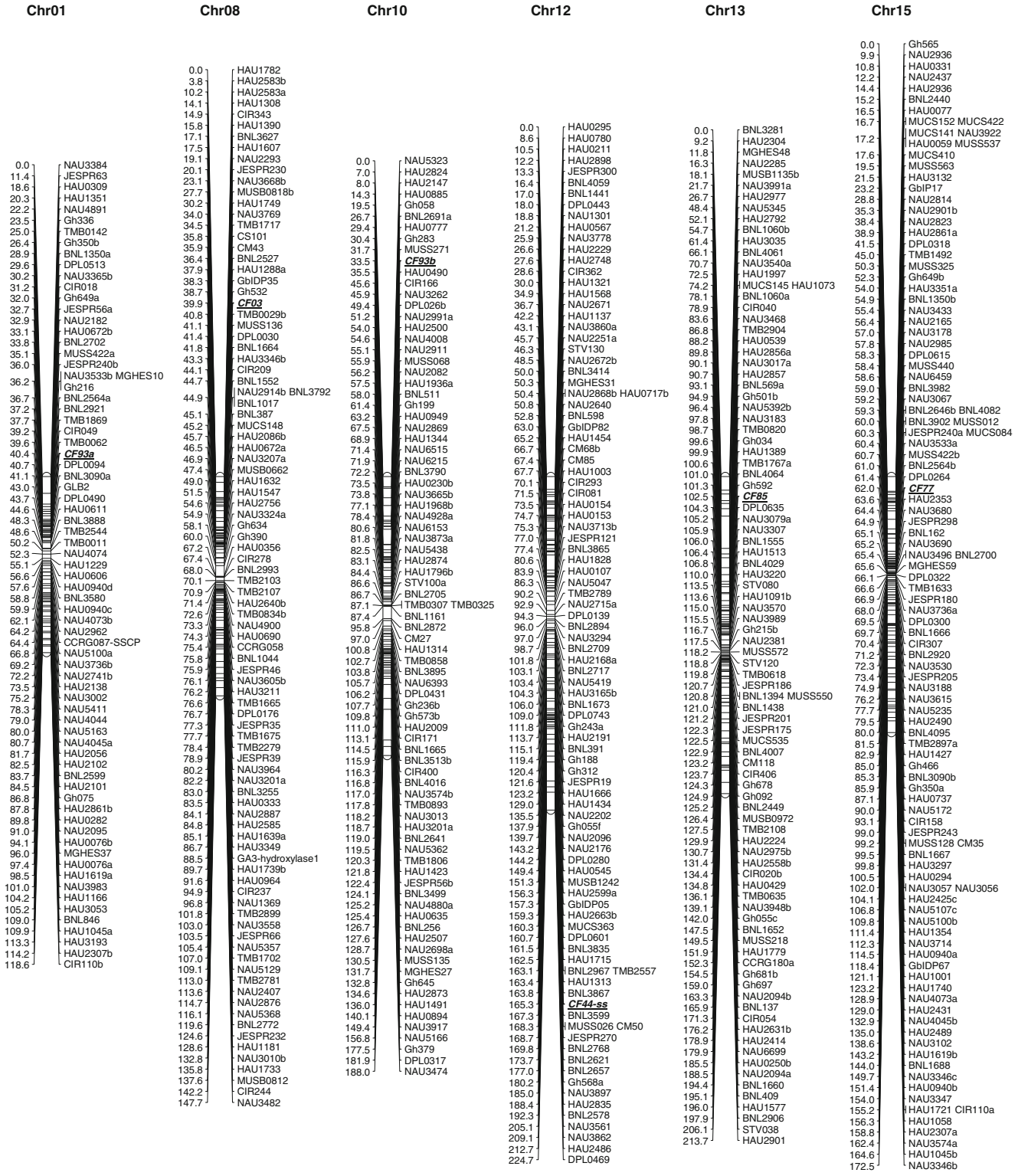

Figure 4. Chromosome distribution of functional markers derived from TDFs (transcript-derived fragments). Functional markers are underlined, italic and bold.

that there are similar expression regulation mechanisms in Emian22 and 3-79.

Among the 110 assembled unique sequences, only 14 sequences (12.73%) were found to match cotton ESTs. Although EST sequencing and microarray can identify thousands of genes involved in cotton fibre development, these methods cannot cover all the genes. On the contrary, although cDNA-SRAP differential display isolated hundreds of

differently expressed genes, the majority of them were novel ones. Annotation of TDFs showed that these TDFs were classified into GO catalogues described in other reports (Yuan *et al.* 2011). The results indicate that cDNA-SRAP differential display can not only identify genes as other methods can, but is also an effective tool to isolate novel genes.

Molecular markers were developed to genetically map these TDFs; however, very low polymorphism was detected between the parents, and few loci were mapped. One reason is that the BC_1 mapping population cannot map Emian22 (recurrent parent) dominant loci; the other reason, possibly the more important one, is that the transcript difference may not produce difference at the DNA level, which indicates that the difference of fibre quality between *G. hirsutum* and *G. barbadense* was mainly derived from transcript difference.

In conclusion, this study not only gave us an overview of transcript difference during fibre development between *G. hirsutum* and *G. barbadense*, but also isolated novel different genes which can be used as candidate genes for further study to confirm their function during fibre development and to be applied in cotton genetic improvement.

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