

RESEARCH ARTICLE

Construction of an EST-SSR-based interspecific transcriptome linkage map of fibre development in cotton

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Abstract

Quantitative trait locus (QTL) mapping is an important method in marker-assisted selection breeding. Many studies on the QTLs focus on cotton fibre yield and quality; however, most are conducted at the DNA level, which may reveal null QTLs. Hence, QTL mapping based on transcriptome maps at the cDNA level is often more reliable. In this study, an interspecific transcriptome map of allotetraploid cotton was developed based on an F₂ population (Emian22 × 3-79) by amplifying cDNA using EST-SSRs. The map was constructed using cDNA obtained from developing fibres at five days post anthesis (DPA). A total of 1270 EST-SSRs were screened for polymorphisms between the mapping parents. The resulting transcriptome linkage map contained 242 markers that were distributed in 32 linkage groups (26 chromosomes). The full length of this map is 1938.72 cM with a mean marker distance of 8.01 cM. The functions of some ESTs have been annotated by exploring homologous sequences. Some markers were related to the differentiation and elongation of cotton fibre, while most were related to the basic metabolism. This study demonstrates that constructing a transcriptome linkage map by amplifying cDNAs using EST-SSRs is a simple and practical method as well as a powerful tool to map eQTLs for fibre quality and other traits in cotton.

[Liu C., Yuan D. and Lin Z. 2014 Construction of an EST-SSR-based interspecific transcriptome linkage map of fibre development in cotton. *J. Genet.* **93**, 689–697]

Introduction

Cotton is one of the most important crops in the world, providing natural fibre used in many facets of daily life. The most extensively cultivated cotton species are the allotetraploid *Gossypium hirsutum* and *G. barbadense*, which account for ~95 and 2% of total production worldwide (National Cotton Council, <http://www.cotton.org>). *G. hirsutum* and *G. barbadense* are known for their high yield and high fibre quality, respectively (Pang *et al.* 2012). The contrasting traits of two species make them ideal for studying the genetic basis of yield and fibre quality in cotton (Mei *et al.* 2004; Lacape *et al.* 2005, 2010; He *et al.* 2007; Yu *et al.* 2013). However, since yield and fibre quality in cotton are complex quantitative traits, studies on the genetic basis of these traits rely on genetic linkage maps.

The first interspecific linkage map was constructed with restriction fragment length polymorphism (RFLP) markers

(Reinisch *et al.* 1994). However, a shortage of RFLP probes restricts worldwide use. Fortunately, the development of PCR-based markers, especially simple sequence repeats (SSRs), has increased rapidly. There are now 17,448 publicly available SSR markers in cotton (<http://www.cottongen.org>), which facilitate linkage map development and QTL mapping in cotton worldwide. EST-SSRs account for nearly half of all SSRs. Several high-density interspecific linkage maps (Rong *et al.* 2004; Yu *et al.* 2011; Zhao *et al.* 2012; Fang and Yu 2012) and intraspecific linkage maps have been developed in cotton. In addition, many QTL mapping studies for yield and fibre quality have been conducted with both interspecific (Mei *et al.* 2004; Lacape *et al.* 2005, 2010; He *et al.* 2007; Yu *et al.* 2013) and intraspecific populations (Ulloa *et al.* 2005; Zhang *et al.* 2005, 2009, 2012; Shen *et al.* 2006; Wang *et al.* 2006; Qin *et al.* 2008; Wu *et al.* 2009; He *et al.* 2011; Liu *et al.* 2012; Sun *et al.* 2012). These studies contribute much to the understanding of the complexity of genetic control of cotton yield and fibre quality.

Recently, the focus on QTL mapping has shifted to the transcriptional level in cotton. Liu *et al.* (2009) applied

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Keywords. cotton; EST-SSR; transcriptome linkage map; fibre development.

cDNA-amplified fragment length polymorphisms (AFLPs) to construct fibre transcriptome groups at the secondary cell wall (SCW) thickening stage, based on an interspecific backcross (BC₁) of *G. hirsutum* × *G. barbadense*. They mapped 78 transcript-derived fragments (TDFs) into eight transcriptome groups, and detected two significant QTLs, FS1 and FS2, which explained 16.08 and 15.87% of the fibre strength variance, respectively. Liu *et al.* (2011) constructed a transcriptome map based on cDNA-AFLPs, using an immortalized F₂ (IF₂) population of the cotton hybrid Xiangzhamian 2 (*G. hirsutum*). A total of 302 TDFs were mapped onto 26 linkage groups, and 71 QTLs for yield and yield component traits were detected, based on four environments, with 13 QTLs identified in at least two environments. Claverie *et al.* (2012) used quantitative cDNA-AFLP to monitor variation in the expression level of cotton fibre elongation and secondary cell wall thickening transcripts in a population of interspecific *G. hirsutum* × *G. barbadense* recombinant inbred lines (RILs). Two-thirds of the normalized intensity ratios of TDFs were mapped between 1 and 6 eQTLs.

Genomewide transcriptome maps form the foundation of gene mapping and cloning as well as comparative genomics. Usually, transcriptome maps are constructed using cDNA-AFLPs since direct mapping of transcripts with the cDNA-AFLP technique is a rapid and practical method of direct mapping of expressed genes (Brugmans *et al.* 2002). Transcriptome maps have been successfully constructed in *Arabidopsis* and potato (Brugmans *et al.* 2002; Ritter *et al.* 2008) as well as in cotton (Liu *et al.* 2009, 2011; Claverie *et al.* 2012) by cDNA-AFLP. The cDNA-AFLP technique is high-throughput, however, the procedure is complex. cDNA-SRAP has been applied for the construction of a transcriptome map in *B. oleracea* (Li *et al.* 2003); this technique is similar to but simpler than cDNA-AFLP.

Compared to these two techniques, constructing transcriptome maps using EST-SSRs is also a practical method of directly mapping expressed genes. Although EST-SSRs are developed from ESTs, EST-SSRs are typically applied in DNA research as they are simpler and easier to work with. In fact, EST-SSRs can be directly used to amplify cDNA to construct SSR-based transcriptome linkage maps. There are many EST-SSRs in different plants, with more than 8000 EST-SSRs in cotton (<http://www.cottongen.org>). Thus, EST-SSRs are a valuable resource for constructing transcriptome maps. Generally, EST-SSRs were first applied in DNA-based genetic maps, implying that they have been mapped to chromosomes. When they are applied in transcriptome maps, the linkage groups can easily be assigned to corresponding chromosomes. However, most TDFs from cDNA-AFLPs and -SRAPs have not been mapped to chromosomes, since it is hard to assign them to chromosomes (Brugmans *et al.* 2002; Li *et al.* 2003; Ritter *et al.* 2008; Liu *et al.* 2009, 2011).

In this study, we utilized the cDNA samples obtained from developing fibres from an F₂ population of Emian22 × 3-79

at five days post anthesis (DPA) as templates to explore the possibility that a transcriptome map could be constructed by amplifying cDNA using EST-SSRs. We have shown that this practical and simple method for constructing transcriptome linkage maps will facilitate eQTL mapping in cotton, which will help to analyse the genetic basis of economically desirable traits at the transcriptional level.

Materials and methods

The mapping parents were the *G. hirsutum* cultivar 'Emian22' and the *G. barbadense* accession '3-79', which are described in the previous work with the BC₁ mapping population (Yu *et al.* 2011). To construct a transcriptome linkage map, a new F₂ population was developed. The F₂ population (~200 individuals) was planted in the experimental field of Huazhong Agriculture University, Wuhan, Hubei, China in 2009. However, only 69 plants were used to construct the map as it was possible to obtain enough samples for RNA extraction from these plants. In the previous study, it was found that the differences in developing fibres at five DPA were obvious between the mapping parents (Liu *et al.* 2013). Therefore, RNA was extracted from developing fibres at five DPA using the method described by Zhu *et al.* (2005). First-strand cDNA was synthesized using 3 µg of RNA from each sample, following the protocol provided with the Superscript[®] III RT kit (Invitrogen, Carlsbad, USA).

Polymorphism screening

All the markers used in this study were obtained from the previously published BC₁ linkage map (Yu *et al.* 2011), which have been mapped to chromosomes. Since the markers on the BC₁ map included gSSRs and EST-SSRs, the EST-SSRs were directly used to screen for polymorphisms in the cDNA of developing fibres at five DPA; the sequences of the gSSRs were blasted against the cotton ESTs to identify SSRs derived from the transcribed sequences. Next, these SSRs were used to screen for polymorphisms.

PCR amplification and electrophoresis

Polymerase chain reaction (PCR) was conducted in 20 µL volume containing 25 ng DNA, 0.2 µmol L⁻¹ forward primers, 0.2 µmol L⁻¹ reverse primers, 1× buffer, 1.5 mmol L⁻¹ MgCl₂, 0.3 mmol L⁻¹ dNTPs and 0.5 units of *Taq* polymerase. The PCR profile consisted of an initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and an extension at 72°C for 1 min, with a final incubation at 72°C for 10 min. First, the amplification products obtained with all of the primers were genotyped using

Table 1. Polymorphisms of different SSRs in developing fibres at five DPA between mapping parents.

SSR	Primer	Total	Primers with products	Primers with polymorphism	
EST-SSRs	HAU	460	186 (40.4%)	95 (20.7%)	
	NAU	648	327 (50.4%)	179 (27.6%)	
	MUSS	36	11 (30.7%)	5 (13.9%)	
	MUCS	16	9 (56.3%)	8 (50.0%)	
	MGHES	19	10 (52.6%)	8 (42.1%)	
	STV	20	5 (25.0%)	2 (10.0%)	
Transcript-derived gSSRs	JESPR	4	2 (50.0%)	1 (25.0%)	
	BNL	16	6 (37.5%)	3 (18.8%)	
	CM	1	1 (100%)	1 (100%)	
	TMB	14	5 (35.7%)	1 (7.1%)	
	CIR	1	1 (100%)	0 (0)	
	DPL	21	6 (28.6%)	0 (0)	
	Gh	8	0 (0)	0 (0)	
	MUSB	6	1 (16.7%)	0 (0)	
	Total		1270	570 (44.9%)	303 (23.86%)

Table 2. Basic information on the F₂ transcriptome linkage map based on developing fibres at five DPA.

Linkage group	Chromosome	Length (cM)	Total loci	Average distance	Largest gap (cM)
1	Chr01	78.53	8	9.82	16.46
2	Chr02	47.38	4	11.85	33.65
3	Chr03	76.10	10	7.61	20.67
4	Chr04	115.23	10	11.52	22.44
5	Chr05	132.74	15	8.85	23.59
6	Chr06	33.16	6	5.53	16.08
7	Chr06	30.58	3	10.19	15.57
8	Chr07	30.03	2	15.02	30.03
9	Chr08	56.63	9	6.29	14.06
10	Chr08	28.16	3	9.39	23.74
11	Chr09	36.12	2	18.06	36.12
12	Chr09	50.74	4	12.69	26.74
13	Chr09	22.18	2	11.09	22.18
14	Chr10	18.8	2	9.40	18.81
15	Chr10	50.81	5	10.16	14.39
16	Chr11	108.68	16	6.79	19.75
17	Chr12	57.02	6	9.50	21.05
18	Chr13	68.79	7	9.83	17.02
A _T -genome		1041.68	114	9.14	36.12
19	Chr14	104.41	11	9.49	24.13
20	Chr15	64.14	15	4.28	11.58
21	Chr16	25.92	2	12.96	25.92
22	Chr17	24.43	3	8.14	12.75
23	Chr18	82.83	8	10.35	25.27
24	Chr19	92.42	15	6.16	11.56
25	Chr20	52.53	6	8.76	19.85
26	Chr21	116.19	17	6.83	32.31
27	Chr22	74.77	9	8.31	17.78
28	Chr23	43.72	5	8.74	15.36
29	Chr24	88.86	18	4.94	20.49
30	Chr25	33.25	3	11.08	18.14
31	Chr25	2.33	2	1.17	2.33
32	Chr26	91.34	14	6.52	13.00
D _T -genome		897.04	128	7.01	36.12
Total		1938.72	242	8.01	36.12

the SSR analysis protocol on 6% denaturing polyacrylamide gels at room temperature (Lin *et al.* 2005). Next, the monomorphic PCR products were separated based on

single-strand conformation polymorphisms (SSCPs) on 6% nondenaturing polyacrylamide gels at a constant 8W for ~16 h at 4°C.

Transcriptome linkage map construction

After polymorphism screening, all the polymorphic markers were used to genotype the entire F₂ population. If a primer detected multiple loci, letters (a, b, c...) were assigned to the loci according to the descending fragment size. The linkage map was constructed with JoinMap 3.0 (Stam 1993) using a logarithm of odds (LOD) threshold of 4.0 and a maximum recombination fraction of 0.4. Map distances were calculated in centi-Morgans (cM) using the Kosambi mapping function (Kosambi 1944). Linkage groups were assigned to

the corresponding chromosomes using mapped SSRs (<http://www.cottongen.org>).

Functional annotation of SSR-ESTs

The SSR sequences were functionally annotated using Blast2GO (Conesa et al. 2005; Götz et al. 2008) with default parameters. First, blastx analysis was carried out with an E-value of 10⁻⁵ against the nr protein database in GenBank; and the sequences with >80% identity were retained. Next, GO-mapping, annotation analysis, InterPro Scan and

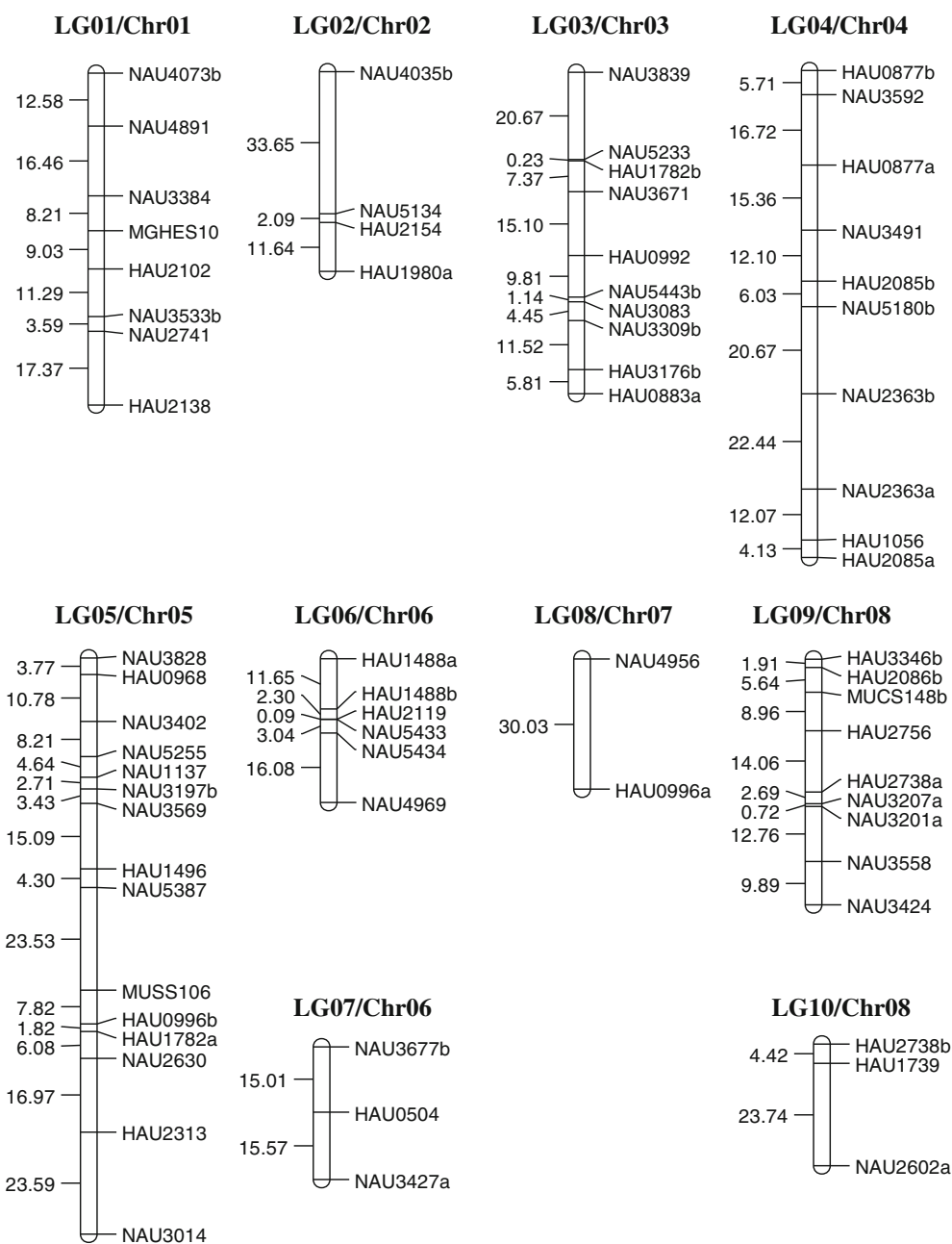


Figure 1 (continues)

InteProScan GOs were performed. Finally, GO-Slim (<http://www.geneontology.org/GO.slims.shtml>) was then carried out to acquire specific GO terms.

Results and discussion

Marker polymorphism

In this study, the mapping parents were the same as those used to construct the DNA-based linkage map (Yu *et al.*

2011). The EST-SSRs in the DNA-based linkage map were directly used to construct the transcriptome map. To map more markers, genomic SSRs in the map were blasted against the cotton ESTs to identify transcribed markers. Finally, 1270 transcript-derived SSRs were included in this study (table 1). The samples used for transcriptome map construction were cDNAs from developing fibres at five DPA, as it was confirmed that a great number of TDFs differed between the mapping parents at this stage (Liu *et al.* 2013). After polymorphism detection, only 303 primers (23.86%) showed

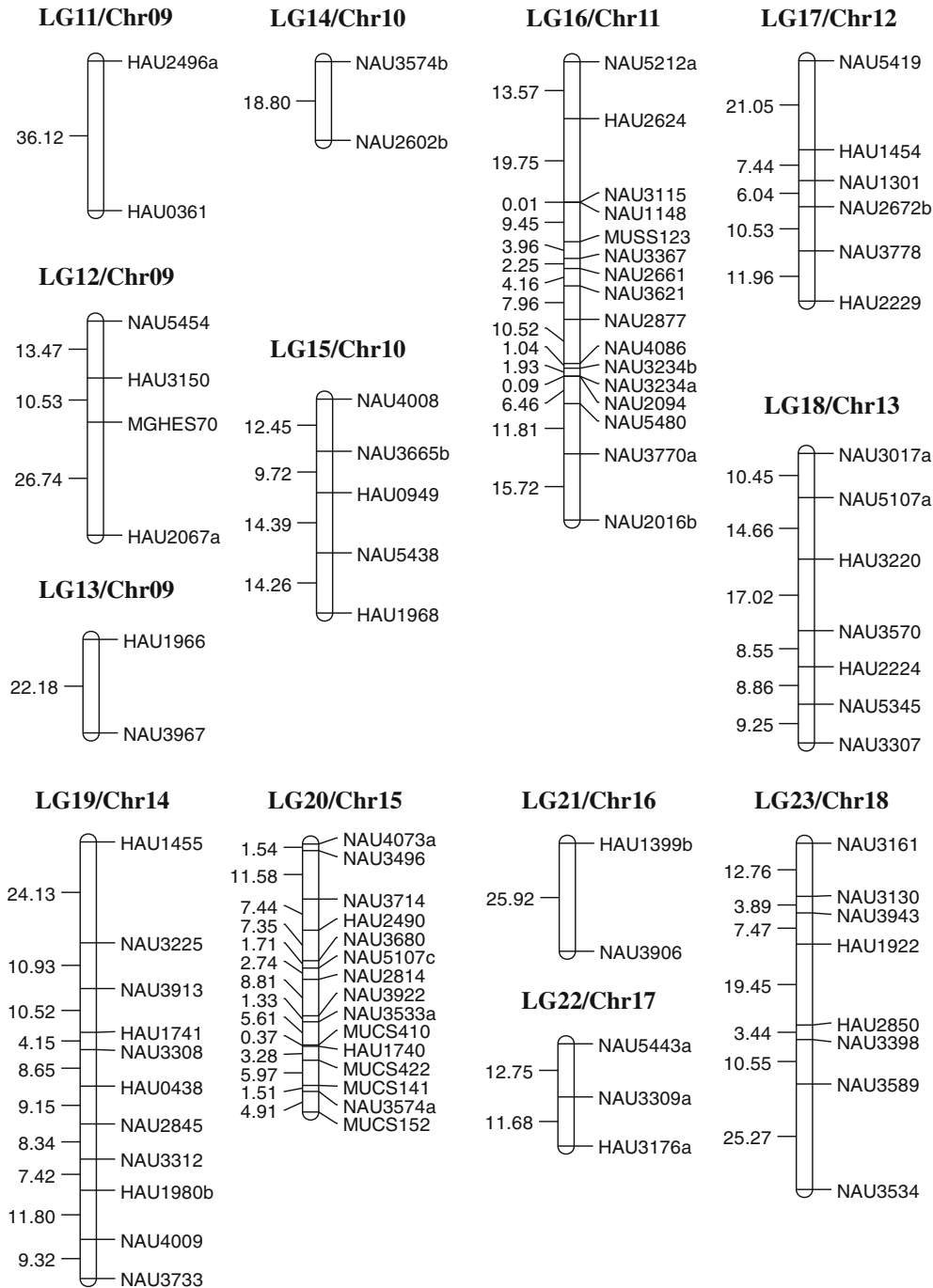


Figure 1 (contd)

polymorphism, 267 (21.02%) produced amplification products, but lacked polymorphism, and 700 (55.12%) did not amplify the target (table 1) The failed amplification is likely to be the reason as these SSRs could not target tissue-specific transcripts in developing fibres at five DPA.

Transcriptome linkage map construction

The cDNA samples obtained from the F₂ population were genotyped with the 303 polymorphic primers; however, some primers generated ambiguous gel bands and were discarded in subsequent analyses. Subsequently, 244 primers produced clearly scorable gel bands with 279 polymorphic loci; among them, 58 (20.79%) were 3-79 dominant loci, 18 (6.45%) were Emian22 dominant, and 203 (72.76%) were codominant loci. Generally, SSRs are codominant. The corresponding genes of the codominant markers are all expressed in the parents. However, variations in the

microsatellite region could occasionally cause alterations in the translated products, which could result in trait variations. The corresponding genes of dominant markers are only expressed in one parent, which could alter the gene network of the trait and result in trait variations.

After linkage analyses, 242 loci were mapped into 32 linkage groups, with 37 remaining loci unmapped. The total length of the transcriptome linkage map was 1938.72 cM (table 2; figure 1). The longest linkage group was 132.74 cM with 15 loci (LG05/Chr05), while the shortest linkage group was 2.33 cM with two loci (LG31/Chr25); generally, the average length of a linkage group was 60.58 cM. The average marker interval distance was 8.01 cM, while it varied from 0.01 (LG16/Chr11) to 36.12 cM (LG11/Chr09). The A_T genome included 18 linkage groups with a total of 114 loci; the total length was 1041.68 cM, and the average marker interval distance was 9.14 cM. Comparatively, the D_T genome included 14 linkage groups with a total of 128 loci;

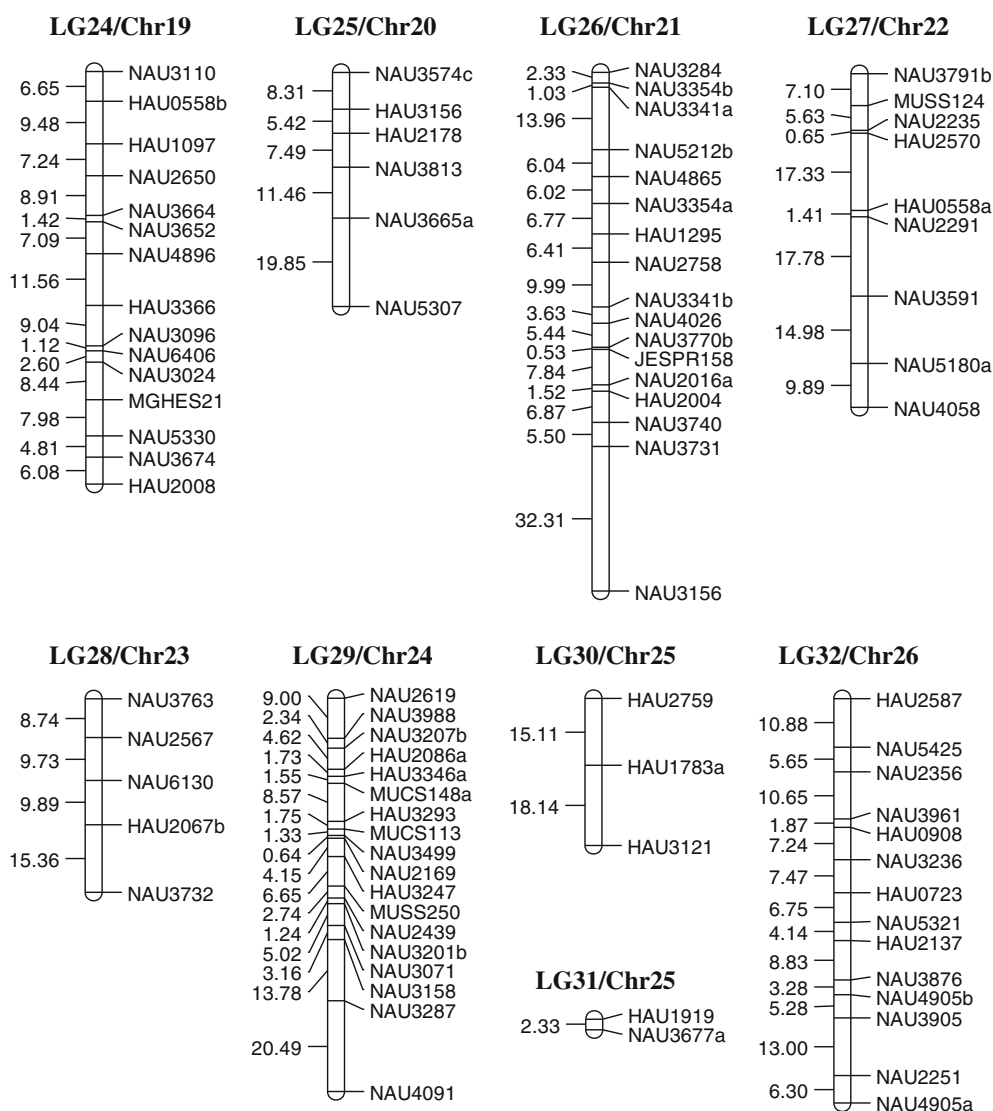


Figure 1. Interspecific transcriptome linkage map of fibre development in cotton constructed using EST-SSRs.

Table 3. Distribution of dominant markers in the transcriptome map.

Chr.	Marker	Chr.	Marker	Chr.	Marker	Chr.	Marker
01	HAU2102	07	NAU4956	14	NAU3733	19	NAU4896
01	NAU3384	08	NAU2602a	15	HAU1740	20	NAU3574c
01	NAU4891	09	NAU5454	15	MUCS141	20	NAU5307
03	HAU0992	10	NAU3574b	15	MUCS152	22	NAU3591
03	NAU3083	10	HAU0949	15	MUCS410	22	NAU3791b
03	NAU5233	10	NAU4008	15	MUCS422	22	NAU4058
05	NAU2630	11	NAU2661	15	NAU3496	23	NAU6130
05	NAU3197b	11	NAU3115	15	NAU3714	24	NAU2169
05	NAU3569	11	NAU3234a	15	NAU3922	24	NAU3071
05	NAU5255	11	NAU3234b	15	NAU3574a	24	NAU3158
05	NAU5387	12	HAU2229	18	HAU1922	24	NAU3287
06	HAU1488b	12	NAU3778	18	NAU3161	26	HAU2587
06	HAU2119	13	NAU3017a	19	HAU1097	26	HAU0723
06	NAU3427a	13	NAU3307	19	HAU2008	26	NAU3961
06	NAU3677b	14	HAU0438	19	MGHES21	26	NAU4905a

the total length was 897.04 cM and the average marker interval distance was 7.01 cM. The genomewide distribution of EST-SSRs implied that the fibre development in cotton involves genes distributed throughout the entire genome.

We found that dominant markers were absent on Chr02, Chr04, Chr16, Chr17, Chr21 and Chr25 (table 3). Obviously, there were more chromosomes lacking dominant markers in the D_T genome than in the A_T genome; however, there were more dominant markers overall in the D_T genome, indicating that the dominant markers were clustered on particular chromosomes of the D_T genome, especially Chr15. The uneven distribution of EST-SSRs on the chromosomes implied that different chromosomes played different roles in developing fibres at five DPA. This result also provided evidence for the existence of tissue-specific transcriptomes.

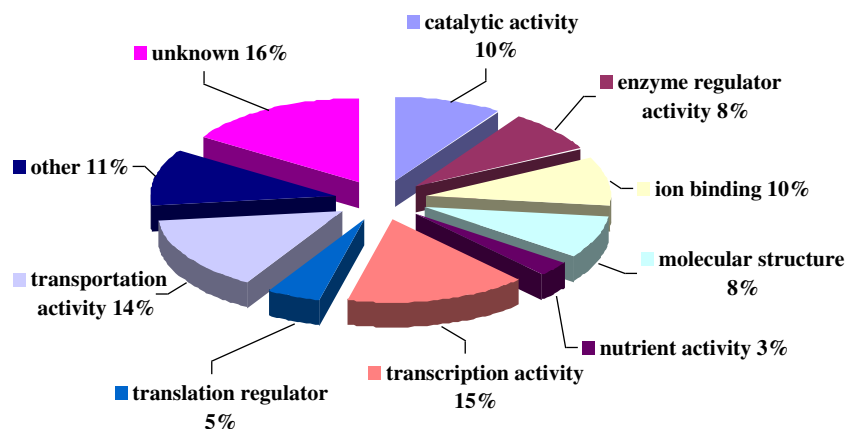
This map is suitable for mapping eQTLs. Unfortunately, most of the cotton bolls were sampled for RNA extraction to construct the transcriptome map; thus, not enough bolls remained to phenotype the fibre quality. However, when transcriptome maps are constructed from permanent populations, it is feasible to map eQTL to understand the genetic basis

of cotton fibre traits at the transcriptional level. We can also compare the eQTLs with previous QTLs based on DNA markers to uncover those reliable QTLs.

Functional annotation of SSR-ESTs

After blasting against the nr protein database, 53 of the 242 SSR-ESTs with >80% identity were functionally annotated, and they were classified into 10 categories (figure 2). Except for the 16% with unknown function, the top functions were transcription activity (15%) and transportation activity (14%). Some SSR-ESTs were involved in fibre differentiation (e.g. NAU3495), fibre initiation (e.g. NAU3496) and fibre elongation (e.g. MUSS250). These results were in accordance with the sampling phase (developing fibres at five DPA) as five DPA is the overlapping period of fuzz cell differentiation, lint initiation and elongation (Lee *et al.* 2007).

In conclusion, an EST-SSR-based interspecific transcriptome linkage map of fibre development in cotton was

**Figure 2.** Functional annotation of mapped SSR-ESTs.

constructed. This innovative new tool for constructing transcriptome linkage maps can be easily used to assign linkage groups to chromosomes and facilitate eQTL mapping in plants.

Acknowledgement

This work was financially supported by the National Basic Research Program (no. 2011CB109303 and 2010CB126001).

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Received 3 December 2013, in final revised form 24 April 2014; accepted 20 May 2014

Unedited version published online: 24 June 2014

Final version published online: 22 September 2014