Molecular Mechanisms of Fiber Differential Development between *G. barbadense* and *G. hirsutum* Revealed by Genetical Genomics

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Abstract

Cotton fiber qualities including length, strength and fineness are known to be controlled by genes affecting cell elongation and secondary cell wall (SCW) biosynthesis, but the molecular mechanisms that govern development of fiber traits are largely unknown. Here, we evaluated an interspecific backcrossed population from *G. barbadense* cv. Hai7124 and *G. hirsutum* acc. TM-1 for fiber characteristics in four-year environments under field conditions, and detected 12 quantitative trait loci (QTL) and QTL-by-environment interactions by multi-QTL joint analysis. Further analysis of fiber growth and gene expression between TM-1 and Hai7124 showed greater differences at 10 and 25 days post-anthesis (DPA). In this two period important for fiber performances, we integrated genome-wide expression profiling with linkage analysis using the same genetic materials and identified in total 916 expression QTL (eQTL) significantly (P<0.05) affecting the expression of 394 differential genes. Many positional cis-/trans-acting eQTL and eQTL hotspots were detected across the genome. By comparative mapping of eQTL and fiber QTL, a dataset of candidate genes affecting fiber qualities was generated. Real-time quantitative RT-PCR (qRT-PCR) analysis confirmed the major differential genes regulating fiber cell elongation or SCW synthesis. These data collectively support molecular mechanism for *G. hirsutum* and *G. barbadense* through differential gene regulation causing difference of fiber qualities. The down-regulated expression of abscisic acid (ABA) and ethylene signaling pathway genes and high-level and long-term expression of positive regulators including auxin and cell wall enzyme genes for fiber cell elongation at the fiber developmental transition stage may account for superior fiber qualities.

Introduction

Cultivated tetraploid cotton yields approximate 97% natural fiber used widely by the textile industry. The two tetraploid species, extra-long staple cotton (*Gossypium barbadense* L.) and Upland cotton (*Gossypium hirsutum* L.) are allotetraploids (2n=4x=52) composed of two ancestral genomes designated A-subgenome (*At*) and D-subgenome (*Dt*), originating from a polyploidy event ~1–2 million years ago [1]. The extra-long staple cotton has superior fiber quality properties such as length, strength and fineness, while Upland cotton is characterized by its short fiber, were found to regulate fiber cell elongation by

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identification of key genes controlling cotton fiber elongation and cell wall biosynthesis [6-16], the molecular mechanism of fiber cell elongation is still not fully understood. Integration of plasmodesmatal gating and expression of sucrose and K⁺ transporters and expansin appear to control fiber cell elongation in a coordinate manner [8], while a fiber-specific β-1,3-glucanase gene (*GhGlu1*) also may play a role in fiber elongation by degrading callose, thus opening the plasmodesmata [9]. Previous microarray analysis has indicated that the genes up-regulated during cell elongation are involved in cell wall structure and biogenesis, formation of the cytoskeleton and energy/carbohydrate metabolism, which corresponds with the high rates of cell expansion occurring during fiber elongation [10]. Several auxin and ethylene related genes have been identified as potential regulators of fiber elongation [11–14], including 1-Aminocyclopropane-1-Carboxylic acid Oxidase (*ACO*) genes which are responsible for the ethylene promote fiber cell elongation in vitro [14]. A novel mechanism of modulating cell elongation processes by regulating redox levels was uncovered in recent findings, in which three genes *GAST1-like*, *Cop1*/*BOV2* and *Per1*, highly up-regulated in the A-genome long fiber relative to the F-genome short fiber, were found to regulate fiber cell elongation by...
controlling H₂O₂ levels [15]. Although considerable functional genomics work on cotton fiber development has been carried out [10,13–16], these studies have mainly been conducted with the *G. hirsutum* species and derivative fiber mutants. Very little information has been accumulated on the differences in fiber gene expression between different tetraploid cotton species. Recently, only two reports have focused on the transcriptional changes between *G. hirsutum* and *G. barbadense* fibers using microarrays [17–18]. However, fiber quality genes are less well-characterized, and little is known about underlying biological causes of these differences in cotton fiber qualities.

With the advance of high throughput gene expression profiling technologies, the concept of “genetical genomics” was proposed [19]. In traditional cotton fiber quality quantitative trait locus (QTL) analyses, linkage mapping leads to the detection of genomic regions which are associated with phenotypic variations in the different interspecific populations [20–25]. Genetical genomics employs this same approach, except that the phenotypes are levels in gene expression resulting in the detection of expression QTL (eQTL). Genetical genomics is a new strategy for identifying genes underlying complex phenotypes [19], which can be used to infer the chromosomal positions of thousands of genes and is particularly valuable for species with genomes that are not fully sequenced. This approach has been applied in model plants such as *Arabidopsis* [26–28] and rice [29], and to crops including barley [30], maize [31–33] and wheat [34]. However, there has been not yet been a report in cotton using this method.

Recent advances in microarray technology applied in cotton have provided a high-throughput platform for measuring fiber gene expression. Here, we present the fiber differential gene expression profile between *Gossypium hirsutum* acc. TM-1 and *Gossypium barbadense* cv. Hai7124. The cotton fiber gene expression profiles were integrated with information on genetic markers at two key developmental stages in the interspecific ([TM-1×Hai7124]×TM-1) BC₁ segregating population [35], further combining QTL mapped for fiber qualities and eQTL. This novel approach to dissecting the genetic basis of complex fiber traits in cotton will allow in-depth characterization of certain targeted QTL and functional genes underlying fiber quality properties and deepen our understanding of molecular mechanisms controlling fiber qualities.

**Results**

**Fiber quality performances and QTL mapping**

Fiber quality data collected from (TM-1×Hai7124) TM-1, an interspecific BC₁S₁ mapping population, grown in the field in Nanjing, China in 2003, 2004, 2005 and 2008 confirmed their large variability: mean fiber length (FL) varied from 28 to 33 mm (parents TM-1 and Hai7124 were at 29 and 33 mm on average, respectively), fiber strength (FS) from 27 to 37 g/tex (parents at 30 and 37 g/tex), and fiber micronaire (FM) or fineness values from 3 to 5 (parents at 4.8 and 4.0). There were statistically significant differences between TM-1 and Hai7124 for FL, FS and FM at the P<0.01 level. Hai7124 exhibited longer, stronger and finer fiber (lower micronaire values) traits than did TM-1. The fiber quality traits from the combined four-year results exhibited continuous normal distribution in this interspecific BC₁S₁ population, typical of quantitative traits (Figure 1a–c).

A total of 12 fiber quality QTL were detected by multi-QTL joint analysis [36], of which eleven were commonly detected by the composite interval mapping (CIM) approach [37] using four-year fiber trait data obtained from interspecific backcrossed population (Table 1). A strong QTL for FL (qFL-A11-2) with the highest logarithm of odds (LOD) score was mapped on chromosome A11 (chr. A11), and the alleles from the superior parent Hai7124 at this QTL increased FL. Three QTL for FS were found on chr. A3 and A5; the positive alleles at qFS-A3-1 and qFS-A5-2 were from TM-1, whereas the positive allele at qFS-A3 came from Hai7124. Two major QTL qFM-D1 and qFM-A9 were also detected at the significance level of P<0.05. At the two QTL for fiber fineness, the Hai7124 allele conferred the finer fiber trait (lower micronaire value). In addition, six QTL-by-environment interactions were tagged (Table 1). The QTL for FL and FS were enriched on regions of chr. A3, which contained three QTL. This suggests that pleiotropic effects and/or close linkage may exist in the same region of QTL for fiber qualities.

**Kinetics of fiber growth and interspecific and temporal variations of gene expression in the different species**

To reveal the differential feature of fiber development that may account for fiber qualities, we measured dynamic changes in FL of TM-1 and Hai7124 at different time points (3–38 days post-anthesis, DPA) and observed that the length increased approximately linearly over the first 15–20 DPA in TM-1 and 23–28 DPA in Hai7124 (Figure 1d). Under these growth conditions the maximum elongation rate occurred at 10–15 DPA and decreased abruptly from 15 DPA, but Hai7124 fiber maintained a higher elongation rate than TM-1 between 20–23 DPA (Figure 1e). Finally fiber elongation in TM-1 ceased before 23 DPA, but in Hai7124 lasted until ~33 DPA. Thus, further analyses of transcript accumulation were required to determine the cause of these differences in TM-1 and Hai7124 fiber development.

To investigate differentially regulated genes in the fiber developmental process, we hybridized a cotton fiber cDNA microarray (GPL2610) with RNA samples from TM-1 and Hai7124 fiber at five different developmental time points, 5, 10, 15, 20 and 25 DPA. In all, 15 two-channel slides were carried out using paired cDNA samples from TM-1 and Hai7124 fibers with three replicates at each stage. We extracted the single channel intensity instead of the ratio value from each two-channel hybridization for further analysis, a strategy that was previously reported to be more flexible and valid [38–40]. We compared mRNA expression levels in developing fiber cells between adjacent time points within and between species using the empirical Bayes method in the limma software [41] based on the criterion of false discovery rate (FDR)<0.05 and fold change (FC)≥2 (Figure 2). Within each species, the number of differentially expressed genes (DEGs) was unequally distributed between adjacent time points. The expression changes were biased toward the mid-stage (10–15 DPA), the transition stage from fiber elongation to SCW biosynthesis, with 2,224 (68.6%) and 2,402 (85.3%) differential genes in TM-1 and Hai7124, respectively. Compared with Hai7124, expression changes in TM-1 were biased toward early and later stages of development. In Hai7124, 276 (5 vs. 10 DPA), 94 (15 vs. 20 DPA) and 44 (20 vs. 25 DPA) genes were differentially expressed, whereas in TM-1, 473, 148 and 396 genes were differentially expressed at the respective time points (Fig. 2). When comparing both species at each stage, a total of 106 and 151 genes were up-regulated by 2 fold in TM-1 at 5 and 10 DPA, respectively, while lower numbers (45 and 82) were observed in Hai7124. Similar numbers of up-regulated genes in TM-1 and Hai7124 were found at 15 or 20 DPA. Most noteworthy is the observation that at 25 DPA, a much larger number (232 total) of genes were differentially expressed between TM-1 and Hai7124, with a bias toward up-regulation in Hai7124 (192 in Hai7124 vs. 40 in TM-1). Most of the 192 genes were preferentially expressed during the fiber fast elongation stage.

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**Figure 1**

A summary of fiber QTL analysis for TM-1 and Hai7124 (A–C, FL, FS and FM). **(A)** Box plots showing the distribution of fiber length (FL) of F₂ RILs derived from a backcross of TM-1 and Hai7124. **(B)** Box plots showing the distribution of fiber strength (FS) of F₂ RILs derived from a backcross of TM-1 and Hai7124. **(C)** Box plots showing the distribution of fiber micronaire (FM) of F₂ RILs derived from a backcross of TM-1 and Hai7124.

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**Table 1**

A list of 12 fiber quality QTL detected by multi-QTL joint analysis [36] from (TM-1×Hai7124) TM-1, an interspecific BC₁S₁ mapping population, grown in the field in Nanjing, China in 2003, 2004, 2005 and 2008. The chromosome and region of each QTL are indicated, along with the fiber trait and allele direction at each QTL. The LOD score and genome-wide significance P value are also shown.
Some among these genes have previously been described to regulate fiber elongation [14], such as genes encoding cell wall-loosening enzymes expansin (EXP1) and pectin methylesterase (PME); cell cytoskeleton genes tubulin beta-1 (TUB1) and profilin; and antiauxin-resistant 3 (AAR3). These genes were more highly expressed in G. barbadense than in G. hirsutum at 25 DPA (Figure S1).

This observation was consistent with elongation of Hai7124 fibers which lasted until 33 DPA, but only until 23 DPA in TM-1 (Figure 1d). These results suggest long-term expression of these fiber elongation genes in Hai7124, whereas expression of these genes decreases rapidly at 20–25 DPA in TM-1. Thus, the two periods at 10 and 25 DPA with higher numbers of DEGs were determined to be crucial for identification of fiber genes regulating elongation at the fast elongation stage and determination of G. barbadense fiber continuing elongation or the termination of G. hirsutum fiber elongation at the transition stage.

**Genome-wide linkage analysis of expression profiles**

We extensively investigated gene expression in fiber cells of 66 randomly selected BC1S1 lines as well as the two parents TM-1 and Hai7124 using another cDNA microarray platform (GPL8569) with abundant probes. A total of 13,760 and 13,411 active transcripts at 10 and 25 DPA, respectively, were detected and 142 and 302 differentially expressed genes (DEGs) between TM-1 and Hai7124 further identified (Table S1). Furthermore, 444 gene expression traits in 66 cotton BC1S1 lines were used to conduct eQTL analysis. Out of these 444 DEGs, 394 genes (89%) were detected to have 916 significant eQTL (P<0.05), including 293 and 623 eQTL at 10 and 25 DPA, respectively, by linkage analysis (Table 2, Table S2). Over a fourth of these genes had only one identified eQTL, and the remaining three fourths had two to seven. The median explained variance of expression (R² values) across all 916 eQTL was 17% with a range from 8% to nearly
Table 1. Biometrical parameters of QTL (LOD≥3) affecting quality traits of cotton fiber.

| Traits | QTL   | Pos. LOD | Additive* (a1;a2;a3;a4) | Involving markers | Composite interval mapping |
|--------|-------|----------|-------------------------|--------------------|---------------------------|
| Major  | FL    | qFL-A11-2| 126 7.9 0.42            | BNL2589-330        | 04,05,08b 122.5–125.6 4.5–8.4 0.95–1.51 11–21 |
|        | FS    | qFS-A3-3 | 63.5 3.2 –0.47          | NAU1167-200        | 03b 63.5 3.5* –1.55 10   |
|        | FS    | qFS-A3-2 | 78.3 3.5 –0.48          | NAU3083-175        | 03 78 4.1* –1.64 11      |
|        | FS    | qFS-A5  | 75.3 3.6 0.51          | NAU779-380         | 03b 75 9.1* 3.74 27      |
|        | FM    | qFM-D1  | 74.9 4 0.11            | BNL2646-125        | 27             |
|        | FM    | qFM-D9  | 135.3 3.5 –0.1         | BNL4053-205        | 03b 132.8 10.6* –0.43 27 |
| G x E  | FL    | qFL-A1  | 55.8 4.7 –0.10;0.00;–0.42;0.52 | BNL1355-370      | 05 47.3 3.3 –0.92 8     |
|        | FL    | qFL-A3  | 58.7 5 0.50;0.27;–0.09;0.32 | NAU5444-280      | 03 63.5 4.7* –1.19 13   |
|        | FS    | qFS-A5  | 12.1 3.7 –0.14;–0.03;0.45;–0.28 | NAU3607-200      | 05 12.1 4.6* 1.06 10    |
|        | FS    | qFS-A11-1| 118.3 3.2 –0.47;–0.06;0.10;0.43 | BNL1151-170      | 05,08b 113.6–118.2 2.9–6.4* 1.08–1.31 11–16 |
|        | FM    | qFM-D4  | 27.3 3.6 0.05;0.13;0.01;–0.18 | JESP965-120      | 04b 27.3 2.76 1.25 7    |
|        | FM    | qFM-D12 | 62.9 3.9 –0.05;–0.05;–0.10;0.21 | Y2583            | 06b 62.9 3.59* 0.78 14   |

G x E indicates genotype x environment interactions. FL, FS, and FM were shortening from fiber length, strength, and micronaire.

*Additive effects of QTL-by-environment interaction in each year; a1;a2;a3;a4 represent four year environment interaction additive effects; a positive value indicates the genotype from the parent Hai124 toward increase the value, a negative value indicates the genotype from the TM-1 toward increase the trait value.

The QTL in each year calculated from increased 2147 markers with 0.5-cM intervals throughout for linkage mapping results.

30 genes, their expression values were extracted from 28 k microarrays for linkage mapping. Out of these 30 functional markers, eight (26.7%) agreed with the positions of eQTL within 18 cM (Table S3), which were considered to be regulated in cis, such as the quinate hydroxycinnamoyltransferase (HCT) gene eQTL on chr. A10. The rest most likely were trans-eQTL, such as 28k_275_A03_CS (Ca2+-ATPase) on chr. A11. These results showed that the majority of eQTL in cotton regulated differential expression in a trans-acting manner in this population.

We observed eight genes associated with fiber quality QTL within 20 cM intervals. The genes encoding cell wall structural proteins including kinesin-like protein and alpha-tubulin 10 were mapped in the intervals of QTL qFL-A1 and qFS-A5, respectively; meanwhile, genes for Ca2+-ATPase, NADPH:quinone oxidoreductase (NQR), and non-symbiotic hemoglobin 2 (Hb2) involved in calcium signaling pathway and reactive oxygen species (ROS) metabolism were localized within QTL for fiber fineness, qFM-D12, qFM-D4 and qFM-D1. However, these results are presently limited for precise identification of fiber quality genes on a large scale, especially for mapping fiber quality QTL.

Figure 2. Number of differentially expression genes from interspecific and temporal comparison. A representative image of mature single cell seed trichomes (“cotton fiber”) from cultivar Gossypium hirsutum acc. TM-1 (up) and Gossypium barbadense cv. Hai7124 (down) (bar = 9.5 mm). Microarray analysis was performed for three replicates of each of five stages (represented by boxes) for both accessions. T5-T25 and H5-H25 were indicated microarray samples for TM-1 and Hai7124 at 5, 10, 15, 20, and 25 DPA, respectively. Numbers marked on the line designate the number of genes up-regulated at least 2-fold (FDR<0.01) relative to their adjacent developmental stage, with the total given in brackets ()'. Numbers marked in brackets ( ) denote the numbers of differentially expression genes between TM-1 and Hai7124 at each stage.

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eQTL hotspots

In this study, the eQTL were not evenly distributed across the genome. The eQTL densities for fiber genes and fiber quality QTL across chromosomes are presented in Figure 3. The largest number (80) of eQTL was mapped on chr. A11, but only 9 eQTL were on chr. D1. It is clear that some regions on chr. A1, A3, A6, A8, A9, A11, D5, D11 and D12 had larger numbers of eQTL, as indicated by the thick horizontal lines. Thus, there were specific genomic regions where the eQTL clustered (hotspots), and a nearly 2-fold higher number of hotspots were distributed in the At than in the Dt (Table 3). These eQTL hotspots appeared to be unequally distributed in the cotton subgenomes during specific fiber developmental stages, where the majority of hotspots during the fiber fast elongation were on chr. A5, A10, D11 and D12, while at the SCW synthesis stage, most of the hotspots were on chr. A1, A8, A9, A11 and D5. The results suggested that regulatory control for many cotton fiber genes exist in short chromosomal intervals that are either gene-rich or that recombine infrequently.

At 46 eQTL hotspots, 55% of the 296 eQTL had a positive additive effect for which the Hai7124 allele toward increased expression, and 45% had a negative additive effect (favorable alleles from TM-1) (Table 3). A strong bias (90%) towards favorable effects from Hai7124 alleles was detected for hotspots on chr. A1, A8, A9, A13, D2, D5, D11 and in the telomeric region of chr. A11. There was also a strong bias (90%) towards favorable effects from TM-1 alleles on chr. A11, D5, D12, D13, and in the telomeric region of chr. A9. Interestingly, the number of observed

Table 2. Number of significant eQTL (P<0.05) detected per gene across 444 genes.

| Number of significant eQTL per gene (P<0.05) | 10DPA | Percent | 25 DPA | Percent | All | Percent |
|---------------------------------------------|-------|---------|--------|---------|-----|---------|
| 0                                           | 15    | 10.6    | 35     | 11.6    | 50  | 11.3    |
| 1                                           | 36    | 25.4    | 86     | 28.5    | 122 | 27.5    |
| 2                                           | 42    | 29.6    | 82     | 27.2    | 124 | 27.9    |
| 3                                           | 28    | 19.7    | 51     | 16.9    | 79  | 17.8    |
| 4                                           | 17    | 12.0    | 31     | 10.3    | 48  | 10.8    |
| 5                                           | 3     | 2.1     | 8      | 2.6     | 11  | 2.5     |
| 6                                           | 1     | 0.7     | 7      | 2.3     | 8   | 1.8     |
| 7                                           | 0     | 0.0     | 2      | 0.7     | 2   | 0.5     |
| Total                                       | 142   | 302     | 444    |         |     |         |

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Figure 3. Comparative distributions of eQTL and fiber quality QTL in cotton. The eQTL density for fiber genes across 13 A- (A) and 13 D- subgenome chromosomes (B). The x axis shows eQTL genetic location on chromosomes, and y axis represents the number of eQTL per cM. QTL for fiber length (FL), strength (FS), and micronaire (FM) are indicated in color bars. *indicates main-effect QTL. Detailed comparisons between locations of eQTL and fiber QTL are plotted in the genetic map in Figure S2.
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Table 3. Details of eQTL hotspots detected across all chromosomes.

| Chr | Position/ Interval(cM)* | No. | No. | No. | Positive (Gb%)b | Negative (Gh%)c | Involving TFs*d | Fiber QTL* |
|-----|-------------------------|-----|-----|-----|----------------|----------------|----------------|-----------|
|     | Chr                      | Total| 10 D| 2SD |                 |                |                |           |
| A1  | 42.2–45.3                | 6    | 1   | 5   | 2(33%)         | 4(67%)         | qFL-A1        |           |
|     | 54.2–55.8                | 6    | 1   | 5   | 2(33%)         | 4(67%)         | qFL-A1        |           |
|     | 84.8                     | 5    |     | 5   | 5(100%)        |                |                |           |
| D2  | 43.1–45.4                | 6    | 2   | 4   | 6(100%)        |                | zf-C3HC4      |           |
| A3  | 69.2–73.1                | 5    | 2   | 3   | 2(40%)         | 3(60%)         | qFS-A3-1      |           |
|     | 77.1–78                  | 6    | 1   | 5   | 1(17%)         | 5(83%)         | zf(B-box)     |           |
|     | qFL-A1                   |       |     |     |                |                |                |           |
| A4  | 86.8–89.3                | 6    | 6   |     | 5(83%)         | 1(17%)         |                |           |
| D4  | 90                       | 5    | 1   | 4   | 1(20%)         | 4(80%)         | bZIP           |           |
| A5  | 108.2–112.9              | 5    | 1   | 4   | 4(80%)         | 1(20%)         |                |           |
|     | 139.6–141.6              | 5    | 5   |     | 2(40%)         | 3(60%)         |                |           |
|     | 152.1–156.1              | 5    | 5   |     | 2(40%)         | 3(60%)         |                |           |
| A6  | 103.1–107.1              | 8    | 8   |     | 5(63%)         | 3(38%)         | NAC091        |           |
| A8  | 27.4–31.4                | 5    | 0   | 5   | 5(100%)        |                | RAP2-4        |           |
|     | 37–39.7                  | 14   | 14  |     | 13(93%)        | 1(7%)          |                |           |
|     | 63.2–65.2                | 5    | 1   | 4   | 5(100%)        |                |                |           |
|     | 94.8–96.8                | 5    | 2   | 3   | 3(60%)         | 2(40%)         |                |           |
| D8  | 112.8–114.3              | 6    | 6   |     | 5(83%)         | 1(17%)         | NST1           |           |
| A9  | 0                        | 5    | 5   |     | 5(100%)        |                | Salt tolerance zf |          |
|     | 6–9.8                    | 6    | 1   | 5   | 1(17%)         | 5(83%)         | Salt tolerance zf |          |
|     | 15.3–18.9                | 5    | 5   |     | 1(20%)         | 4(80%)         | AP2; zf(B-box) |           |
|     | 78.1–81.3                | 5    | 1   | 4   | 4(80%)         | 1(20%)         |                |           |
|     | 96.6–98.6                | 7    | 7   |     | 7(100%)        |                | HSP            |           |
|     | 101.5–105.5              | 9    | 9   |     | 8(89%)         | 1(11%)         | NST1;NAC1      |           |
| D9  | 0–2                      | 5    | 1   | 4   | 1(20%)         | 4(80%)         | zf-C3HC4      |           |
| A10 | 42.9–44.9                | 5    | 5   |     | 2(40%)         | 3(60%)         |                |           |
| D10 | 58.6                     | 6    | 1   | 5   | 5(83%)         | 1(17%)         | GRAS           |           |
| A11 | 0–2                      | 10   | 1   | 9   | 10(100%)       |                |                |           |
|     | 53.4–55.7                | 7    | 1   | 6   | 7(100%)        |                | RAP2-4;ERF;bZIP |           |
|     | 64.1–66.7                | 5    | 5   |     | 5(100%)        |                | GRAS; bZIP     |           |
|     | 78.1–81.7                | 5    | 1   | 4   | 5(100%)        |                | GRAS           |           |
|     | 100.5–102.5              | 6    | 6   |     | 1(17%)         | 5(83%)         |                |           |
|     | 104.7–106.7              | 8    | 1   | 7   | 8(100%)        |                |                |           |
|     | 115.5–119.4              | 7    | 2   | 5   | 7(100%)        |                | qFL-A11-1      |           |
| D11 | 14.1                     | 5    | 3   | 2   | 3(60%)         | 2(40%)         |                |           |
|     | 16.1–20.1                | 10   | 10  |     | 10(100%)       |                |                |           |
| A12 | 23–27                    | 6    | 6   |     | 3(50%)         | 3(50%)         |                |           |
| D12 | 35.8–38.8                | 8    | 2   | 6   | 5(63%)         | 3(38%)         | HSP            |           |
|     | 61.8–62.9                | 9    | 7   | 2   | 9(100%)        |                | qFM-D12       |           |
| A13 | 35.3–36.2                | 5    | 5   |     | 5(100%)        |                | NAC091        |           |
| D13 | 96.1–101.1               | 5    | 5   |     | 5(100%)        |                |                |           |
|     | 111.3–114.9              | 5    | 2   | 3   | 2(40%)         | 3(60%)         |                |           |
| At  | 30                       | 187  | 27  | 160 | 98(52%)        | 89(48%)        |                |           |
| Dt  | 16                       | 109  | 36  | 73  | 65(60%)        | 44(40%)        |                |           |
| Total| 46                      | 296  | 63  | 233 | 163(55%)       | 133(45%)       |                |           |
eQTL, whether bias towards Hai7124 or TM-1, in the A-subgenome was greater than those in the D-subgenome (Table 3).

eQTL hotspots can facilitate the identification of underlying biological links. We found that a large proportion of chalcone synthase (CHS) genes that were extensively mapped to the eQTL hotspots regions, involving chr. A5, A11, D10, D11 and D12 during fiber cell elongation, and chr. A1, D8, A9 and D9 during SCW formation. The two largest hotspots related to CHS were on chr. D11 and D12 during fiber elongation, and one large SCW formation hotspot related to CHS was on chr. A1. Some CHS genes were co-localized in the hotspot regions with genes encoding plant hormones, transcription factors and cell wall enzymes, such as responsive to abscisic acid 1B (Rab1B), ACC synthase 6 (ACS6) on A9, NAC secondary wall thickening promoting factor1 (NST1) on D8, Zinc finger (C3HC4-type) family protein on D9, β-1,3-glucanase in the telomeric region of chr. A11, and invertase and polygalacturonase (PG) on D11. These results implied that CHS may regulate fiber cell development through interaction with plant hormones and cell wall enzymes.

A biologically meaningful eQTL hotspot would represent the location of a master transcriptional regulator that controls the expression of a suite of fiber genes acting in the same biological process. We found genes encoding several transcriptional factors in the hotspot regions (Table 3), including AP2/ERF, Zinc finger, GRAS, bZIP, HSP and NAC family transcriptional regulators. Thus, these transcriptional regulators which reside in the fiber quality QTL regions may be crucial in regulating cotton fiber development.

Combining QTL mapped for fiber qualities and eQTL

Association analysis between phenotypic variation and eQTL would be useful in dissecting the genetic factors controlling fiber quality properties. By comparative analysis of eQTL and stable QTL for fiber qualities in the present study (Figure S2), especially for eQTL hotspots within the regions of QTL for qualities (Table 3), we identified some eQTL genes co-localized with QTL for fiber qualities (Table 4), suggesting that they may play a part in mediating the development of fiber phenotypes. In addition, the genes underlying qualities with no functional annotations were also listed in Table S4. Some genes differentially regulated at 10 and 25 DPA were associated with fiber quality properties; most noteworthy, the fiber gene expression variation at 25 DPA predominantly affected fiber quality properties. Two eQTL hotspots on chr. A3 co-localized with qFS-A3-1 and qFS-A3-2 encode proteins primarily involved in oxidation reduction.

It should be noted that the most significant QTL for fiber length and fineness were located on chr. A11 and A9, coinciding with the two highest numbers of eQTL (80 and 70) or hotspots (7 and 6) across all chromosomes. On the same chr. A11 there was a strong bias (>90%) towards positive effects from TM-1 alleles in five eQTL hotspots, whereas, QTL for FL, qFL-A11-1 and qFL-A11-2 with positive effects from longer staple parent Hai7124 alleles increased FL. Co-localizing genes residing in the two QTL included those encoding ACS6, PP2C family proteins, NAC domain protein (NAC1), calmodulin-binding family proteins and calcium-transporting ATPase. These down-regulated genes in Hai7124 underlying genetic and expression loci may play a part in superior FL. There were two eQTL hotspots neighboring qFL-A11-1 that encode proteins involved in oxidoreductase activity, including NADH dehydrogenase, Ca3+-ATPase, ATP6, fatty acid desaturase 8 (FAD8) and alcohol dehydrogenase. Additionally, there was a strong bias (89%) towards positive effects from Hai7124 alleles in two eQTL hotspots on chr. A9 neighboring a main-effect QTL qFM-A9; and this QTL was associated with fineness due to positive effects of alleles from the Hai7124 parent with finer fiber traits. Thus, up-regulated genes in Hai7124 at qFM-A9, such as the genes for dynamin-like protein 4 (ADL4) and NAD/Pd dehydrogenase (ND1), play a positive role in fiber fineness, whereas the down-regulated genes for carbohydrate esterase and polygalacturonase inhibiting protein 1 (PGIP1) may play a negative role in fiber fineness. The eQTL hotspot genes encode proteins, including transcription regulators such as Zinc finger, AP2, NST1 and NAC1, and cell wall enzymes β-1,3-glucanase and glucan endo-1,3-beta-glucosidase-like protein 4 (E13L3). These eQTL hotspots neighboring the QTL for fiber qualities intervals also produced some candidate genes for regulating fiber length or fineness.

Interestingly, some common eQTL genes appeared to associate with different QTL, such as the gene for cellulose synthase-like protein A2 (CSLA02) commonly associated with qFL-A5 and qFL-A11-2. NADH dehydrogenase genes involved in oxidoreductase activity associated with reactive oxygen species (ROS) metabolism were commonly co-localized with QTL for FL, FM and FS. Meanwhile, a suite of genes encoding key CHS involved in flavonoid biosynthesis co-localized with QTL for FL and FM; several protein kinase genes appeared to correlate with FL and FM (Table 4), implying their crucial roles in common molecular events in developmental processes determining these fiber quality traits. In addition, the GAST1-like genes regulating H2O2 levels were mapped in the homoeologous regions of fiber QTL qFS-A3-2 and qFL-A11-2, such as 28k_113_E06_CS and 28k_142_D03_CE.

QRT-PCR analysis for selected fiber genes

Based on eQTL co-location and hotspots, we selected 18 candidate genes to confirm differential expression from our microarray analysis in TM-1 and Hai7124 (Figure 4). For the majority of the tested genes, the qRT-PCR results confirmed the microarray analysis. These genes are primarily involved in flavonoid biosynthesis, plant hormones and calcium signaling pathways, transcription factors, and cell wall polysaccharides biosynthesis or modification. Several genes were preferentially expressed in the fiber fast elongation stage (10 DPA) (Figure 4A-D). The expression of cell wall loosening enzyme gene PME was observed to decrease sharply at SCW synthesis in TM-1, but
decreased slowly in Hai7124. Interestingly, the expression of most of the tested genes in TM-1 increased rapidly when cell elongation ceased at 25 DPA, whereas in Hai7124 they rose slowly (Figure 4E–N). These genes were up-regulated during 25 DPA in TM-1 but were repressed in Hai7124 that produced longer fibers, suggesting that these genes, such as those encoding Rab1B, ACS6, NAC and Zinc finger (C3HC4-type) family transcription factor, β-1,3-glucanase, play a specific role in preventing fiber elongation or promoting SCW synthesis. The expression of three genes encoding glucan endo-1,3-beta-glucosidase-like protein 4

Table 4. Identifying eQTL genes co-localized with QTL for fiber qualities.

| Stages | Co-localized QTL | Array ID | Gene Name | Putative function |
|--------|------------------|----------|-----------|------------------|
| 10 DPA | qfL-A1           | 28k_093_F01 | Transferase family protein | - |
|       | qfL-A3           | 28k_094_F09 | XH/XS domain-containing protein | - |
|       | qfL-A5           | 28k_045_H06 | Serine-threonine protein kinase | Catalytic activity |
|       | qfL-A11-1        | 28k_172_A10 | NADH dehydrogenase subunit 1 | Oxidation reduction |
|       | qfS-A3-2         | 28k_196_G03 | CYP71A16 | Oxidoreductase activity |
|       | qfM-A9           | 28k_110_B01 | Probable carbohydrate esterase | Hydrolase activity |
|       | qfM-A9           | 28k_208_A09 | Polygalacturonase inhibiting protein 1 | Protein binding |
|       | qfM-D12          | 28k_047_B08 | CHS | Flavonoid biosynthesis |
|       | qfM-D12          | 28k_073_A01 | CHS | Flavonoid biosynthesis |
|       | qfM-D12          | 28k_181_G05 | Chromatin remodeling complex subunit | ATP binding |
|       | qfM-D12          | 28k_257_D01 | CHS | Flavonoid biosynthesis |
| 25 DPA | qfL-A1           | 28k_097_H05 | Arabinogalactan peptide 20 | - |
|       | qfL-A1           | 28k_123_C04 | PB1 domain-containing protein | - |
|       | qfL-A1           | 28k_144_D11 | Speckle-type POZ protein | Protein binding |
|       | qfL-A1           | 28k_152_A03 | Putative GTP-binding protein | Response to gibberellin |
|       | qfL-A1,qfM-D12   | 28k_013_A02 | CHS | Flavonoid biosynthesis |
|       | qfL-A1,qfM-D12   | 28k_079_H10 | CHS | Flavonoid biosynthesis |
|       | qfL-A1,qfM-A11-2 | 28k_166_C10 | Cellulose synthase-like A2 (CSLA02) | Cellulose synthase activity |
|       | qfL-A11-1        | 28k_087_B10 | ABA-responsive protein | ABA signaling pathway |
|       | qfL-A11-1        | 28k_275_A03 | Calcium-transporting ATPase | Calcium ion transport |
|       | qfL-A11-1        | 28k_290_B01 | ACS6 | Ethylene biosynthesis |
|       | qfL-A11-1        | 28k_298_E02 | Nudix hydrolase homolog 4 (NUDT4) | - |
|       | qfL-A11-1        | 28k_294_H02 | Dehydratase | - |
|       | qfL-A11-2        | 28k_302_B12 | Calmodulin-binding family protein | Calcium ion signaling |
|       | qfL-A11-2        | 28k_098_C10 | NAC domain protein (NAC1) | Regulation of transcription |
|       | qfL-A11-2        | 28k_245_C05 | PP2C family protein | Protein dephosphorylation |
|       | qfL-A11-2        | 28k_144_D04 | LRR-RLK | - |
|       | qfL-A11-2        | 28k_211_E10 | FAD-binding domain-containing protein | Oxidation reduction |
|       | qfL-A11-2        | 28k_288_G01 | ABC1 family protein | Protein phosphorylation |
|       | qfL-A11-2        | 28k_302_B01 | Zinc finger (B-box type) family protein | Zinc ion binding |
|       | qfL-A11-2        | 28k_303_C11 | GDSL-motif lipase | Lipase activity |
|       | qfL-A11-2        | 28k_102_C09 | ADL4 | Response to cadmium ion |
|       | qfL-A11-2        | 28k_121_B04 | ATP binding/phenylalanine-tRNA ligase | - |
|       | qfL-A11-2        | 28k_158_D06 | 24-sterol C-methyltransferase (SMT2-1) | BR biosynthetic pathway |
|       | qfL-A11-2        | 28k_260_H12 | NAD(P)H dehydrogenase (ND1) | Oxidation reduction |
|       | qfL-A11-2        | 28k_233_G07 | Probable disease resistance protein | ATP binding |
|       | qfL-A11-2        | 28k_082_G03 | Abscisic acid-responsive HVA22 protein | ABA signaling pathway |
|       | qfL-A11-2        | 28k_209_H05 | TCP family transcription factor | Regulation of transcription |

These transcripts of differentially expressed genes were co-localizing with phQTLs in the overlapping intervals, reference Figure S2 in detail. Gene Ontology (GO) annotations and searches of the literature were conducted to identify putative function of differentially expressed genes.

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Figure 4. qRT-PCR validation of selected differentially expressed genes. The x axis represents two developmental periods (10 and 25 DPA), and y axis indicates the relative expression value from qRT-PCR (A1-R1) and signal intensity from microarray (A2-R2) in TM-1 and Hai7124 fibers. Data shown are the means of three biological replicates. Error bars indicate ± SD.
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Discussion

*Gossypium barbadense* produces superior fibers than *G. hirsutum*, yet the regulatory mechanisms governing its difference in fiber qualities are not well known. In this study, the dynamic analysis of fiber growth and gene expression variation among different time points between *G. barbadense* and *G. hirsutum* suggested two respective periods (10 and 25 DPA) that were crucial for further identification of fiber quality genes. Furthermore, we integrated large-scale microarray-based gene expression profiling with linkage analysis in cotton and found 916 eQTL in each stage (10 and 25 DPA) after assessing genome-wide significance. These eQTL represent a large source of functional genes for the fiber QTL that were mapped from the combined four-year data in this study. The fiber length measurements suggested that significant differences between *G. barbadense* and *G. hirsutum* occurred at the overlapping period of elongation and SCW formation, which was longer in *G. barbadense* (19–33 DPA) than that in *G. hirsutum* (19–23 DPA) (Figure 5). This result agrees with previously reported results, and the duration of transition stage may determine cotton fiber quality properties [2–4,6,9,17]. At this transition stage, *G. hirsutum* is known to have a higher rate of cellulose deposition than *G. barbadense* [3–4], although *G. barbadense* maintains a higher rate of fiber elongation than *G. hirsutum*. Interestingly, a large proportion of differentially regulated genes at 25 DPA in this study appeared to be associated with fiber length, strength and fineness (Table 4), which enabled us to further elucidate the molecular mechanisms controlling differential fiber length in *G. hirsutum* and *G. barbadense* (Figure 5).

Molecular basis of prolonged fiber elongation in *G. barbadense*

Plant hormones and cell wall enzymes play a prominent role in mediating fiber cell elongation. Two sets of microarray platforms showed similar results with significantly increasing trends of up-regulated genes in *G. barbadense* from 10 to 25 DPA (82 vs. 192 and 29 vs. 113, respectively) when compared with *G. hirsutum*. The

Figure 5. Molecular mechanisms of differential cotton fiber length in *G. hirsutum* and *G. barbadense*. *G. barbadense* showed a longer transition stage with overlapping fiber elongation and secondary cell wall (SCW) synthesis than *G. hirsutum*. Metabolic pathways were differentially regulated during fiber development, and pectin and cellulose metabolism in particular affected fiber elongation and SCW biosynthesis. In the SCW synthesis stage (represented by 25 DPA), the auxin signaling pathway and pectin modification genes were up-regulated in *G. barbadense* compared with *G. hirsutum*, and the SCW synthesis genes were repressed. In *G. hirsutum* pathways producing cellulose were enhanced, while auxin and pectin modification were repressed. Selected genes were also analyzed by qRT-PCR as in Figure 4. Red represents up-regulated in TM-1, green represents up-regulated in Hai7124. Underlined genes were differentially expressed only at 10 DPA. The abbreviations used are: day post-anthesis (DPA); chalcone synthase (CHS); quinate hydroxycinnamoyltransferase (HCT); antiauxin-resistant 3 (AAR3); auxin response factor 2 (ARF2); auxin-responsive up-regulated in Hai7124. Underlined genes were differentially expressed only at 10 DPA. The abbreviations used are: day post-anthesis (DPA); calmodulin (CaM)-binding protein; ethylene-responsive transcription factor (ERF); protein phosphatase 2C family protein (PP2C); abscisic acid (ABA); responsive to abscisic acid 1B (Rab1B); NADPH oxidase (NOX); Cell wall enzymes: pectin methyl esterase (PME); pectate lyase (PEL); polygalacturonase (PG); polygalacturonase inhibiting protein 1 (PGIP1); glucan endo-1,3-beta-glucosidase-like protein 4 (E13L3); Zinc finger (B-box type) family protein and dynamin-like protein 4 (ADL4); Zinc finger (C3HC4-type) family protein (ZF (C3HC4)); UDP-glucose pyrophosphorylase (UGP); Cellulose synthase (CEL); NAC secondary wall thickening promoting factor1 (NST1). doi:10.1371/journal.pone.0030056.g005
expressions of fiber cell elongation related genes decreased slowly in *G. barbadense*, suggesting fiber elongation related genes had long-term expression and supported a longer duration of elongation in this cotton species at the transition stage. Of these, auxin signaling pathway and pectin modification genes antiauxin-resistant 3 (*AARS*) and pectin methylesterase (*PME*) preferentially expressed at the fast elongation stage were up-regulated in *G. barbadense* compared with *G. hirsutum* at 25 DPA and showed similar time-course expression patterns (Figure S1). Previous reports have indicated that auxin promotes fiber initiation and early stages of elongation [7,11,13,42], but little is known about their roles at the transition stage. The cell-wall loosening enzyme PME was repressed in the gain-of-function Aux/IAA mutant (aux1-1/aia17-1) for auxin resistant 3 in *Arabidopsis* [43]. Recent studies suggest that genes regulating pectin play key roles in controlling fiber cell elongation, comparative proteomics confirmed that biosynthesis of pectic precursors was important for cotton fiber and *Arabidopsis* root hair elongation [16]; pectin synthesis genes were the most significantly differentially expressed categories during fiber elongation, and the expression levels of pectin modification genes showed high correlations with specific fiber properties [18]; transgenic modifications also confirmed that GhPEL encoding pectate lyase positively regulated fiber length [44]. These data indicate that auxin may regulate fiber cell elongation through pectin modification (Figure 5).

Turgor-driven cell expansion is required for cotton fiber elongation at the transition stage. The two novel genes encoding glucan endo-1,3-beta-glucosidase-like protein 4 (*E13L3*) involving callose binding activity, which were highly expressed in *G. barbadense* fibers at 25 DPA, led to an increase in callose accumulation to regulate plasmodesmata closure [9,45]. By contrast, we found several genes for beta-1,3-glucanase, which degrade callose to regulate plasmodesmata opening, that had low levels of expression in *G. barbadense* fibers [9]. These two gene families control duration of plasmodesmata closure to sustain high turgor for a longer period, consistent with longer fiber elongation stage in *G. barbadense* than in *G. hirsutum*. Therefore, these results suggest auxin signaling pathway and cell wall enzymes provide a basis for *G. barbadense* fiber to sustain elongation.

**Molecular basis for the early termination of fiber elongation in *G. hirsutum***

Ethylene and abscisic acid (ABA) may regulate cotton fiber cell development through increasing H$_2$O$_2$ levels. Comparative analysis of eQTL and QTL for fiber qualities identified that ethylene, ABA and calcium signaling pathway genes mapped in eQTL hotspots co-localized with the major QTL for FL, such as genes encoding ACS6, PP2C and ABA-responsive proteins, CaM-binding protein and Ca$^{2+}$-ATPase. A large proportion of ethylene and ABA signaling pathway genes sharply increased from 10 to 25 DPA and were significantly up-regulated in *G. hirsutum* fibers at 25 DPA when compared with *G. barbadense* (Figure 4). These over-expressed genes in *G. hirsutum* fibers are associated with H$_2$O$_2$ regulation. Previous studies indicated that both of ethylene and ABA can promote H$_2$O$_2$ production in cotton fiber and *Arabidopsis* [46–47], and ABA is considered a signal of secondary wall thickening during cotton fiber elongation [48]. The present study also showed a GAST1-like gene (28k_113_E06) was preferentially expressed in *G. hirsutum* compared with *G. barbadense* in the SCW stage. GAST1-like (GASA1) is a member of the gibberellin-induced, cysteine-rich protein family previously shown to be induced by H$_2$O$_2$ [49]. These results imply a higher level of H$_2$O$_2$ in *G. hirsutum* fiber cells than *G. barbadense* at 25 DPA, functioning as a developmental signal in the onset of SCW synthesis and the end of cell elongation in cotton fibers [15,50]. Thus, ethylene and ABA may trigger the onset of SCW synthesis and the end of cell elongation in cotton fibers through increasing H$_2$O$_2$ levels.

A previous study showed that ethylene promotes cotton fiber cell elongation [14], which was based on experiments using low concentrations of ethylene in the ovule culture at the early stage of fiber elongation. Other studies have shown that low concentrations of ethylene can modulate low-level H$_2$O$_2$ production that is important for cell elongation in *Arabidopsis* and cotton [15,46,51]. Our study also showed that two GAST1-like genes (28k_142_D03 and 28k_153_G08) which were preferentially expressed at 10 DPA in *G. hirsutum*, may promote fiber elongation through modulating H$_2$O$_2$ levels. 28k_142_D03 was mapped within an eQTL hotspot associated with a major QTL for FS on A3, and 28k_153_G08 was mapped within 2 cM with a gene encoding glutamate de-carboxylase 4 (GAD4) involved in the calcium signaling pathway. H$_2$O$_2$ is generated by ABA-induced activation of NADPH oxidase and mediates activation of the Ca$^{2+}$ channel pathway in *Arabidopsis* [47,51]. In this study, several Ca$^{2+}$ signaling pathway genes were up-regulated in *G. hirsutum* than *G. barbadense* at 25 DPA, implying high levels of Ca$^{2+}$ in *G. hirsutum* fiber cells, which can increase the cell wall rigidity by binding de-esterified pectin and restrict further wall expansion [52]. The metabolic pathways at the transition stage in *G. hirsutum* streamed into high-rate cellulose synthesis, and fiber elongation trended towards cessation. Our results showed that the expressions of Zinc finger (C3HC4-type) and NAC family transcription factors sharply increased during *G. hirsutum* fiber SCW synthesis (Figure 4K-M; Zinc finger (C3HC4-type) family transcription factors also regulate cellulose synthesis via oxidation of the zinc-binding domains [53]. NST1 is a key regulator of the formation of secondary walls in *Arabidopsis* [34]. This cascade of transcription factors regulates expression of genes to promote the biosynthesis of secondary walls. These results support that ABA and ethylene may inhibit fiber elongation and enhance SCW synthesis through regulating high levels of H$_2$O$_2$ and calcium at the transition stage, which is a possible basis for the termination of fiber elongation.

Most of the genes in flavonoid biosynthesis pathway were up-regulated in TM-1 during fiber elongation. Of these, a large percentage of CHS genes were extensively mapped in the regions of eQTL hotspots on chr. A1, A5 and A11 associated with significant QTL for fiber length. Previous studies demonstrated that flavonoids accumulation act as negative regulators of auxin transport when the gene encoding quinate HCT in lignin synthesis is repressed [55–56]. Since ethylene also modulates flavonoid accumulation [57], CHS and ethylene therefore can affect auxin transport by enhancing flavonoid accumulation, which may negatively regulate fiber cell elongation. These results support down-regulated expression of these fiber elongation related negative regulators in *G. barbadense* during the transition stage likely help fiber cells to maintain auxin levels, which is another basis for *G. barbadense* fiber cell to sustain elongation.

In conclusion, the high-level and long-term expression of auxin regulators, pectin modification and turgor related genes may promote continuing fiber cell elongation by cell wall loosening and enlargement at the transition stage; and down-regulation of the ABA and ethylene signaling pathway and SCW biosynthetic gene expression in *G. barbadense* helps the fiber cells to maintain the high elongation rate for sustained elongation (Figure 5). These may be the two major causes for fiber cell sustained elongation at the transition stage. These molecular mechanisms may facilitate the longer duration of the transition stage in *G. barbadense* and ultimately determine superior fiber quality properties such as fiber length, strength and fineness.
To our knowledge this study is the first to integrate developmental genetics and genomics of a single cell type in plants, and which provides an opportunity to better understand how complex plant hormones-signaling pathways are integrated to control cotton fiber qualities. Further functional validation of the currently large percentage of genes with unknown functions in fiber QTL will provide novel clues for a fiber cell model involving elongation or cellulose biosynthesis. With the completion of cotton genomic sequencing projects in the near future and further localization of genes, many more cis-acting eQTL genes residing in fiber QTL should be obtained from the data gained in this study, which will provide a rich resource for studying genes underlying fiber qualities. Genetic improvement of commercial cotton cultivar fiber quality properties is expected to be achieved using these novel gene resources or loci in this study by genetic transformation or marker assisted selection breeding programs.

Materials and Methods

Plant materials

TM-1 is a genetic standard accession of *G. hirsutum* developed in the USA, and Hai7124 is a commercial *G. barbadense* Verticillium-resistant cultivar with superior fiber length, fineness and strength. All BC1 (TM-1/Hai7124/TM-1) plants were grown in the field during the summer seasons and transferred to the greenhouse during the winter seasons at Jianggu Cotton Research Station of Nanjing Agricultural University (JCRSNAU), Nanjing, China. All BC1 plants were self-pollinated to produce replicate individuals from the BC1 mapping population, which enabled us to perform four independent field trials using the same genotypes. The two parents (TM-1 and Hai7124) and 138 BC1/S1 lines were grown during the summer seasons at JCRSNAU, Nanjing, China in 2003, 2004, 2005 and 2008. Fiber samples of four years were harvested from at least 15 plants of the BC1/S1 lines, and the following fiber quality traits were evaluated by using the HVI Spectrum (Zellweger Uster Corp., Switzerland): 2.5% fiber length (FL, mm), fiber strength (FS, cN/tex) and micronaire reading (FM).

Fiber samples of TM-1 and Hai7124 at 5, 10, 15, 20 and 25 DPA in 2006 were used for 12 k microarray hybridizations, and that from TM-1, Hai7124 and 66 randomly selected out of 138 BC1/S1 lines at 10 and 25 DPA in 2008 for 28 k microarray hybridizations. These samples were collected individually from the 15 plants in the morning (10:00–11:30 AM), and the fiber cells with the exception of those at 5 DPA were carefully dissected from the ovules excised from each boll. All harvested fiber materials were immediately frozen in liquid nitrogen and stored at −70°C.

Fiber length measurements

To reveal the dynamics of fiber development, seeds of TM-1 and Hai7124 at 5, 10, 15, 20, 23, 28, 33 and 38 DPA were chosen to determine the FL using a previously reported method [58]. Six sample replicate data were processed using Microsoft Excel.

RNA isolation, amplification and labeling

Three equal-volume pools of each sample of TM-1 and Hai7124 were used for extraction of total RNA. Total RNA was extracted from 5 DPA ovules and 10 to 25 DPA fibers of TM-1 and Hai7124 using a modified hot-borate method [59]. Total RNA was extracted from the 10 and 25 DPA fibers of TM-1, Hai7124 and the 66 BC1/S1 lines using a CTAB method [60]. The RNA was concentrated in isopropyl alcohol, followed by purification using the NucleoSpin® RNA Clean-up Kit from MACHEREY-NAGEL (MN, Germany) and finally checked for quantity and purity. Total RNA was reverse transcribed into double-stranded cDNA using the CbcScript enzyme. The cRNA was then synthesized from the second-strand cDNAs with the T7 Enzyme Mix, and the amplified cRNA products were purified using the RNA Clean-up Kit. The cDNA was synthesized with random primers from cRNA using the CbcScript II enzyme. The cDNA products were purified using a PCR NucleaseSpin Extract II Kit (MN, Germany) and labeled with fluorescent dye (Cy3 and Cy5-dCTP) using the Klenow Fragment Polymerase from the cRNA Amplification and Labeling Kit.

Cotton fiber cDNA microarray hybridization

The 11,692 probes of the 12 k cotton fiber cDNA microarray (GPL2610) and 29,184 probes of the 28 k cotton cDNA microarray platform (GPL569) were from 5–10 DPA and −3–25 DPA Upland cotton (*G. hirsutum* L. cv. Xuzhou 142) ovules and fibers, respectively. The Cy3 and Cy5-labeled cDNA were resolved in 80 μl hybridization solution (3×SSC, 0.2% SDS, 5× Denhart’s, 25% formamide) and denatured at 95°C for 3 min prior to loading onto the microarray. The slides were hybridized at 42°C overnight and transferred to fresh containers with Wash Solution 1 (0.2% SDS, 2× SSC) at 42°C for 5 min and then immersed in Wash Solution 2 (0.2× SSC) at room temperature for 5 min. All microarrays were scanned with the LuxScanTM scanner, and the array image signals were translated into numeric signals with the LuxScanTM 3.0 software (both from CapitalBio Corp., China). All 12 k cotton cDNA microarray data from this work have been deposited to a MIAME compliant database at the National Center for Biotechnology Information (NCBI) GEO database (GSE18028, GSM450751 to GSM450764).

Statistical analysis

Statistical analyses of differential gene expression measured in the cDNA microarrays were carried out with open source R software packages available as part of the Bioconductor project (http://www.bioconductor.org). Considering that global gene expression changes may exist across different developmental stages, we applied a linear normalization method to normalize individual channel data. The normalized signal intensity values in the 15 microarray slides from the 12 k platform hybridized with RNA prepared from three biological replicate samples for each developmental stage were further analyzed using the empirical Bayes method in the LIMMA package [41], and they were filtered according to the following criteria: (i) exclude genes with low-intensity signal levels (raw signal value <400); (ii) assignment present in 3 repeats from expression ratios; and (iii) log-transformation results in a fold change greater than 1 with Bonferroni adjusted P-value <0.05.

A total of 72 slides including six of the two parents and 66 of the BC1/S1 population at 10 and 25 DPA were obtained from the 28 k microarray platform. The raw signals from the microarrays were normalized linearly based on 72 slides after applying background subtraction. Analysis of DEGs was performed using the empirical Bayes method in the LIMMA package [41], and they were filtered according to the following criteria: (i) assignment present in 3 repeats from normalized signal; (ii) exclude genes with low-intensity signal levels (raw signal value <250); and (iii) log-transformation results in a fold change greater than 1 with expected P-value of less than 0.05 by LIMMA [41]. Normalized data from the 66 BC1/S1 samples at each fiber developmental stage were used for further linkage analysis. Gene Ontology (GO) annotation was used to infer biochemical pathways involved in genes by Blast2GO (http://www.blast2go.de).
Genetic and Expression QTL Analysis in Cotton

Linkage and data analysis
A complete linkage map for all 26 chromosomes was constructed using major microsatellite markers [35]. We selected 654 markers from a complete linkage map, including 2,147 markers with 5-CM intervals between markers on 26 chromosomes. The CIM analysis was implemented using Windows QTL Cartographer 2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). LOD scores were calculated at 2-CM intervals throughout the genome for each gene expression. QTL Cartographer was used to establish genome-wide significance using the permutation test, and at least 1,000 permutations were used to establish empirical P-values (0.05). QTL for FL, FS, and FM at each year were calculated using the CIM approach [37]. QTL and QTL-by-environment interactions for FL, FS and FM with combined four-year data were evaluated using multi-QTL joint analysis under the framework of penalized maximum likelihood [36].

QRT-PCR analysis
For qRT-PCR validation, additional biological samples were collected from TM-1 and Hai7124 at 10 and 25 DPA. qRT-PCR analyses were performed on three biological replicates from three independent RNA samples. First-strand cDNA was synthesized from 1 µg total RNA using the M-MLV reverse transcriptase (Promega, USA) according to the manufacturer’s instructions. Gene-specific primers were designed according to EST sequences in microarray (Table S6). The other seven DEGs in cotton fiber that had been obtained from 1-mu cellulose biosynthesis in developing cotton fibers. Int. J. Dev. Biol. 41: 633-640.

Molecular mapping
The 23 locus-specific PCR primers were designed according to the EST sequences in microarray (Table S6). The other seven genes including the redundant sequences of SSR-ESTs had been mapped previously in our lab [35]. PCR amplifications were performed as follows: at 95°C for 10 min, 30 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 90 s, followed by a final extension at 72°C. The polymorphic loci were integrated to our backbone linkage maps using JoinMap 3.0 [61].

Supporting Information
Figure S1 Temporal expression variation in G. hirsutum and G. barbadense fibers. (DOC)
Figure S2 Chromosomal co-localizations of eQTL and QTL for fiber qualities. (DOC)
Table S1 List of all differentially expressed genes at 10 and 25 DPA. (XLS)
Table S2 Details of 916 significant eQTLs in cotton genome. (XLS)
Table S3 Comparison of fiber quality and eQTL mapping with chromosomal locations mapping for 30 probe sets. (DOC)
Table S4 Identifying candidate genes associated to fiber quality properties. (DOC)
Table S5 Primers used in this study for qRT-PCR analysis. (DOC)
Table S6 Gene specific primers used in this study for mapping. (DOC)

Author Contributions
Conceived and designed the experiments: TZZ. Performed the experiments: XDC WZG. Analyzed the data: XDC BLI YC LLZ. Contributed reagents/materials/analysis tools: YMZ XLS. Wrote the paper: XDC TZZ.

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