Transcriptome analysis reveals gene expression associated with fuzz fiber initiation regulated by high-temperature in *Gossypium barbadense*

Gongmin Cheng

College of Agronomy, Northwest A&F University; Cotton Research Institute, Chinese Academy of Agricultural Sciences

Longyan Zhang

Cotton Research Institute, Chinese Academy of Agricultural Sciences; College of Agronomy, Hebei Agricultural University

Hengling Wei

Cotton Research Institute, Chinese Academy of Agricultural Sciences

Hantao Wang

Cotton Research Institute, Chinese Academy of Agricultural Sciences

Jianhua Lu

Cotton Research Institute, Chinese Academy of Agricultural Sciences

Shuxun Yu (✉ ysx195311@163.com)

Chinese Academy of Agricultural Sciences Cotton Research Institute <https://orcid.org/0000-0002-9715-3462>

Research article

Keywords: *Gossypium barbadense*, high temperature, fuzz initiation, RNA-seq, gene expression

Posted Date: March 26th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-18232/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: *Gossypium barbadense* L. is the most important renewable source of textile fiber. Cotton fiber cell initiation and elongation are often affected by various environmental stimulus, such as high temperature. However, little is known about the underlying mechanisms of temperature regulating the fuzz fiber initiation.

Results: In the present study, phenotypic observation revealed that high temperature (HT) accelerated the fiber development, improved fiber quality and induced fuzz fiber initiation. It has been proved that the fuzz fiber initiation was inhibited by low temperature (LT), and 4 days post-anthesis (DPA) was the key stage for fuzz fiber initiation. Based on comparative transcriptome analysis, a total of 43,826 differentially expressed genes (DEGs) were identified, of which 9,667 were involved in both fiber development and temperature response with 901 transcription factor genes and 189 genes related to plant hormone signal transduction. Further analysis of gene expression patterns revealed that 240 genes were involved in fuzz fiber initiation. Functional annotation revealed that the candidate genes related to fuzz initiation were significantly involved in asparagine biosynthetic process, cell wall biosynthesis and stress response. Furthermore, the expression trends of sixteen selected genes from the RNA-seq data were almost consistent with the results of qRT-PCR results.

Conclusions: Our study revealed several potential candidate genes and pathways that related to fuzz fiber initiation induced by high-temperature and provided a new view of temperature-induced tissue and organ development in *Gossypium barbadense*.

Background

The genus *Gossypium* includes two allotetraploid cultivars, *G. hirsutum* L. (AD₁) and *G. barbadense* L. (AD₂), whose annual yield is more than 90% of the cotton fiber production. *G. hirsutum* is widely cultivated all over the world due to its high yield, while *G. barbadense* is not high in yield but prized because of its excellent fiber quality [1]. Mature cotton seeds are covered with two different types of fibers, lint and fuzz, both of which are mainly composed of cellulose. The development of cotton fibers can be divided into four overlapping stages: fiber initiation, elongation, thickening of secondary cell walls, and dehydration [2]. Lint fiber initiation occurs from - 3 DPA to 3 DPA [3]. With the help of scanning electron microscope (SEM) and cotton fiber mutants, the fuzz fiber initiation stage was determined to be 4 DPA [4, 5]. The first three stages of fiber development are hypersensitive to environmental stress [6]. Extreme temperature and other abiotic stresses during fiber development can significantly reduce cotton fiber yield and quality, and may even lead to falling bolls and squares [7–9].

In plants, studies of trichomes and root hairs in *Arabidopsis thaliana* has greatly expanded our understanding of cotton fiber initiation and elongation. Numerous genes have been found to be involved in trichome initiation in this model plant. In *Arabidopsis*, it has been widely believed that the MBW transcriptional complex (GL3/EGL3-GL1-TTG1) determines the fate of trichome cells by inducing the

expression of GL2 [10]. In addition, GIS2, ZFP8, ZFP6 and ZFP5 were found to promote the expression of MBW complex members in Arabidopsis [11–13]. There are many similarities between cotton fibers and Arabidopsis trichomes in the regulatory mechanism of initiation and elongation development, which has been proven by some cotton homologs. GhMYB25-like and GhMYB25 are two R2R3 MYB genes that regulate fiber initiation and elongation. GhMYB25-like plays a major role in regulating fiber initiation and development. Silencing GhMYB25-like will inhibit cotton lint and fuzz fiber initiation development [14]. Overexpression of GhMYB25 in cotton increases the number of trichomes on leaves and the fibers number on ovule epidermis at anthesis [15]. As another R2R3 MYB transcription factor, GhMYB109 also specifically expressed during lint initiation and elongation development [16]. As a homeodomain-leucine zipper (HD-ZIP) transcription factor, GhHOX3 is revealed to play a major role in regulating fiber elongation. Overexpression of GhHOX3 increases fiber length, while silencing the gene reduces fiber length [17]. Overexpression of another HD-ZIP transcription factor, GhHD1, increased the number of initiating fibres, but had not other adverse effects on leaf hairs [18]. Cotton PROTODERMAL FACTOR1 gene (GbPDF1) is mainly expressed during fiber initiation and early elongation stages, and silencing of GbPDF1 will retard fiber initiation [19].

Cotton fiber initiation and elongation are not only dependent on their genetic specificity, but also regulated by environmental factors and endogenous hormones. Auxin, gibberellin and brassinolide have long been known to play important roles in plant cell expansion and elongation. As another phytohormone, ethylene is widely studied in fruit ripening, dormancy release, and stress response. In addition, ethylene also has been found to play an important role in regulating root hair and hypocotyl development [20, 21]. Auxin, gibberellin, brassinolide and ethylene have positive effects on the fiber development, while abscisic acid and cytokinin inhibit the fiber cell development [22–24]. Jasmonic acid increases the number of leaf trichomes in Arabidopsis by promoting the expression of GL3 [25, 26]. In cotton, JA-related metabolism promotes fiber initiation [27]. In addition to phytohormones, Ga^{2+} and H_2O_2 also regulate the fiber cells development [28].

Temperature is a major environmental factor affecting plant growth and development. Plants of different species require different growth temperatures, and different developmental stages or developing organs of the same plant also require favorable temperatures. High temperature stress is an abiotic stress common to all crops from subtropical origin. High temperature stress during flowering period can cause male sterility in some crops, such as rice and cotton [29, 30]. Some molecular mechanisms of plant response to high temperature have been revealed, including the response of transcription factors and heat shock proteins, calcium ion and reactive oxygen species signal transduction, and response of endogenous plant hormones such as auxin and gibberellin [31–33]. These studies provide a model for temperature-regulated development of specific tissues or organs, such as the development of cotton fibers in high temperatures.

Most *G. barbadense* varieties have a low density of fuzz fibers, which is different from most *G. hirsutum* varieties whose seeds surface are completely covered by fuzz fibers. It is often observed that the fuzz density of the same *G. barbadense* cultivar varies with different environments, and the fuzz density in

Xinjiang and Hainan Province is lower than that in Anyang City [34]. In this study, 121 *G. barbadense* cultivars were used for fuzz density evaluation. The temperature of full-bloom stage in three main growing sites in China was investigated. Accession L7009 was considered sensitive to environmental stimulus. We also verified the influence of temperature on fuzz fiber initiation of L7009 and when the fuzz fiber initiation occurred, and examined the effect of different temperatures on fiber quality. In order to understand the transcriptional regulation mechanism of temperature on the early development of lint fibers and fuzz fiber initiation, we conducted comparative transcriptome on ovules of L7009 in different temperatures. Then we performed functional enrichment analysis and co-expression analysis of differentially expressed genes from different profiles. These findings in this study extend our understanding of how temperature affects the development of cotton fibers, and also provide valuable data for studying the mechanism of temperature-regulated fuzz fiber development.

Results

Temperature regulates fuzz fiber initiation and affects lint fiber development

To reveal the environmental influence on fuzz and lint fibers development, we investigated fuzz density (FD) and lint fiber quality of 121 *G. barbadense* cultivars in three cities (Korla, Anyang and Sanya). For convenience, we visually graded the fuzz density to eight levels (Additional file 1: Figure S1). In Sanya, 23.97% of the cultivars showed low fuzz density (FD=0.5) and 20.66% showed high fuzz density (FD=3 or 3.5). However, in Korla and Anyang, 15.70% and 2.48% of the cultivars showed low fuzz density, while 29.75% and 60.34% showed high fuzz density, respectively (Fig. 1a). This suggests that the environment of Sanya is more likely to reduce fuzz density, and the environment of Anyang is more likely to increase the fuzz density. We also investigated the relationship between lint quality and fuzz density of 98 cultivars planted in Sanya. Three of the five fiber quality indexes showed an significant positive correlation between fuzz density and lint fiber quality (FL, $r=0.59$; FS, $r=0.61$; FE, $r=0.47$) (Additional file 2: Figure S2). To test whether temperature affects the fuzz density, we investigated the temperature of three environments during the blooming period of three years. The days with minimum temperature above 24°C accounted for 64.6% of the total survey days ($n=96$) in Anyang, while 71.9% of the days ($n=93$) were below 22°C in Sanya (Fig. 1b). Therefore, we inferred that temperature may be the key environmental factor affecting fuzz density of *G. barbadense*. We assumed that high temperature may increase the fuzz density, while low temperature may have the opposite effect on it.

To test our hypothesis, we simulated the ambient temperature of the natural environments (Fig. 1c). It was found that the fuzz density in high-temperature environment (HT, 28~35°C) was significantly higher than that in low-temperature environment (LT, 20~25°C) (Fig. 1d). This indicates that temperature is indeed the decisive environmental factor regulating fuzz initiation, and that high temperature increases the fuzz density. Based on the fact that fuzz density of L7009 was significantly affected by temperature, we designed an experiment to confirm the time when the fuzz initiation occurs. First, by transferring cotton plants from HT to LT (HTL), we found that the fuzz density at 4 DPA (or DT) was significantly

lower than that at 5 DT or higher than that at 2 DT, while there was no significant difference when compared to 3 DT, indicating that 3~4 DPA was an important period for fuzz fiber initiation development (Additional file 3: Table S1; Fig. 2a-b). To make the results more credible, we transported another batch of cotton plants from LT to HT (LTH), and found that the fuzz density at 4 DT was significantly lower than that at 3 DT or higher than that at 6 DT, while there was no significant difference when compared to 5 DT, indicating that 4~5 DPA was also important for fuzz fiber initiation development (Additional file 3: Table S1; Fig. 2c-d). By comparing the fuzz density of HTL and LTH at the same stages, it was found that there was no significant difference between HTL and LTH at 4 DPA, while significant differences between any other stages were found, indicating that 4 DPA was the key stage for fuzz initiation, which was consistent with previous findings in *G. hirsutum* (Additional file 3: Table S1) [5]. For further study, the early and later stages of fuzz fiber differentiation were assigned to 1 and 7 DPA, respectively. With phenotypic observation, we found that high temperature could accelerate fibers initiation and development and increase the fuzz density on seed surface (Fig. 3a-b). In addition, it was found that SFC_n, FS, HGW, FP, SS, Length_w, UQL, MR, FNC, SCNC, FL, SFC_w, Fineness, SCNMS and Micronaire in HT were significantly different from those in LT ($p<0.05$) (Additional file 4: Figure S3; Fig. 3c). Among them, six fiber traits in HT were significantly better than those under LT, and the differences in Length_n and IFC were not significant.

Transcriptome sequencing and comparative analysis of differentially expressed genes between different stages and environments

To reveal the molecular regulation mechanism of ambient temperature on the fuzz fiber initiation and development of L7009, we constructed cDNA libraries from 12 samples of three stages under two environments. The cDNA libraries were then sequenced using an Illumina HiSeq 4000 sequencing platform based on paired-end sequencing. All RNA-seq raw datasets were deposited in the NCBI database with a SRA accession number SUB6753336. We totally obtained 73.43 Gb clean data after mRNA sequencing for 12 samples with at least 6.00 Gb clean data for each sample. In each sample, more than 93.30% of bases score Q30 and above (Table 1). The clean data were then mapped to the reference genome, with the mapping ratio varying from 90.62% to 95.17%. Based on the mapped results, alternative splicing prediction, genetic structural optimization and novel genes discovery were analyzed. Finally, 5,994 novel genes were identified, 5,180 of them are functionally annotated (Additional file 5: Table S2). Based on the alignment results, gene expression analysis was performed. We utilized StringTie to estimate its expression level based on a maximum flow algorithm, and used FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) to measure the transcript or gene expression levels. A total of 63,113 transcripts were obtained with FPKM values > 0 in at least one sample, of which 44,864 were expressed with FPKM values ≥ 1 (Additional file 6: Table S3).

Table 1 Summary of sequencing data for different fuzz fiber developmental stages in high and low temperatures.

Sample	Raw Reads ($\times 10^7$)	Raw Bases ($\times 10^9$)	Clean Reads ($\times 10^7$)	Clean Bases ($\times 10^9$)	GC (%)	Q30 (%)	Mapped Reads (%)
H1-1	4.26	6.38	4.18	6.27	45.94	93.71	92.62
H1-2	4.15	6.22	4.07	6.10	45.63	93.83	94.86
H4-1	4.23	6.34	4.14	6.22	46.19	93.46	94.49
H4-2	4.19	6.28	4.11	6.16	45.81	93.61	95.17
H7-1	4.11	6.17	4.03	6.05	46.13	93.32	94.54
H7-2	4.25	6.37	4.17	6.25	45.80	93.56	94.89
L1-1	4.10	6.16	4.02	6.03	45.25	93.50	91.86
L1-2	4.10	6.15	4.01	6.01	45.54	93.62	90.62
L4-1	4.14	6.21	4.05	6.08	46.98	93.30	92.65
L4-2	4.17	6.25	4.09	6.13	45.76	93.45	94.36
L7-1	4.17	6.25	4.08	6.13	45.92	93.33	95.05
L7-2	4.08	6.11	4.00	6.00	45.84	93.56	92.36

The Pearson correlation coefficient between two biologically repeated samples of the same developmental stage in different environments fluctuated between 0.98 and 0.99, and the sample clustering also showed a good correlation between the two biological replicates (Fig. 4a). The results of principal component analysis (PCA) of the 12 samples were also consistent with the above analysis results (Fig. 4b).

Intra-environment and inter-environment comparisons of all expressed genes were performed to reveal the dynamic transcriptional changes during fuzz fiber initiation. In total, 26048 DEGs were identified under the thresholds of absolute $\log_2$ ratio ≥ 1.0 and p -value < 0.01 . In HT, 14,490 DEGs were found at 1 DPA vs 4 DPA (3393 up-regulated, 11,097 down-regulated), 2160 DEGs at 4 DPA vs 7 DPA (1230 up-regulated, 930 down-regulated), and 12,716 DEGs at 1 DPA vs 7 DPA (3628 up-regulated, 9088 down-regulated) (Fig. 4c-d). In LT, 5694 DEGs were found at 1 DPA vs 4 DPA (1473 up-regulated, 4221 down-regulated), 4290 DEGs at 4 DPA vs 7 DPA (1667 up-regulated, 2623 down-regulated), and 17,748 DEGs at 1 DPA vs 7 DPA (3352 up-regulated, 14,396 down-regulated) (Fig. 4c-d). Compared to LT, the HT samples had 2480 DEGs at 1 DPA (951 up-regulated, 1529 down-regulated), 7355 DEGs at 4 DPA (3072 up-regulated, 4283 down-regulated), and 4164 DEGs at 7 DPA (2876 up-regulated, 1288 down-regulated) (Fig. 4c-d). This indicated that the fuzz fiber initiation development is more susceptible to temperature stress at 4 DPA. Of these DEGs, the genes differentially expressed between LT and HT treatments at the same stages were identified as temperature responsive genes, and those identified at different stages

under the same temperature environment were defined as possibly related to fiber development. Therefore, we identified 9667 DEGs that were likely to be involved in both temperature response and fiber development (Additional file 7: Figure S4).

Gene expression trend analysis and PPI network construction

The 9667 DEGs were subjected to hierarchical clustering using Mfuzz package of R software and divided into 9 clusters (Additional file 8: Figure S5), in which 4756 (49.2%) genes with membership ≥ 0.7 were illustrated with a hierarchical clustering heatmap (Fig. 5a). The expression levels of 512 genes in cluster 5 were continuously up-regulated from H1 to H7 and L4 to L7 (Fig. 5b). Genes in clusters 6 (823) and 7 (240) were predominantly expressed at L1 and H4, respectively. According to our previous verification, fuzz fiber initiation occurred at 4 DPA under HT (H4), but didn't occur in low temperature condition. Combined with phenotypic observation, we speculated that the genes in cluster 7 were mainly involved in fuzz fiber initiation induced by high temperature. In contrast, genes in cluster 5 and 6 were involved in lint fiber elongation and initiation under temperature regulation, respectively. However, most of the genes were involved in lint fiber initiation development, and only a small number of genes were related to the fuzz fiber initiation, which indicated that the regulation of lint fiber development is relatively complicated and preferentially.

The 240 genes with membership ≥ 0.7 in cluster 7 were mapped to the STRING database (<https://string-db.org/>) and the PPI network was visualized using the Cytoscape software to investigate the possible interaction between the differentially expressed genes (Fig. 6a). In the PPI network, the node genes with larger degree are defined as hub genes. In the cluster 7, *P5CS1* (GB_D01G2569), *KING1* (GB_A12G2167), *STZ* (GB_D05G2149), *BCAT-2* (GB_A05G2168), *ACX4* (GB_A13G1045), and *HSPRO2* (GB_A10G2743) were defined as hub genes, which were considered as candidate genes involved in regulating fuzz initiation in high temperature.

Gene ontology and KEGG pathway analysis of DEGs

There is a partial overlap in the developmental stages of lint fiber elongation and fuzz fiber initiation. Not only are their underlying gene expression patterns different, but the functions of related genes may also differ significantly. To further understand the functions of the DEGs in the three clusters, GO enrichment for each cluster was performed (Additional file 9: Table S4). To reduce the functional redundancy among GO-terms, the presence of overrepresented GO-terms were obtained using REVIGO program (<http://revigo.irb.hr/>) and visualized using treemap package of R software [35].

The DEGs in cluster 5 were significantly (FDR < 0.05) enriched into 30 GO-terms of biological process, of which the terms involved "fatty acid biosynthetic process", "monocarboxylic acid biosynthetic process", "lipid biosynthetic process", "microtubule-based process" and "intracellular protein transport" were the most significant (Additional file 9: Table S4). Forty significant terms for molecular functions were

enriched, the most significant of which were redundant and involved "structural constituent of cytoskeleton", "GTPase binding", "UDP-glycosyltransferase activity" and "actin binding" (Additional file 9: Table S4). For cell component 16 terms were also enriched and the most significant involved "microtubule", "cytoskeleton" and "membrane" (Additional file 9: Table S4). The DEGs in cluster 6 were significantly enriched into 104 GO-terms of biological process, of which the supercluster terms involved "glycerolipid metabolism", "dephosphorylation" and "protein deacetylation" (Fig. 7). Fifty-one significant terms for molecular functions were enriched, the superclusters of which involved "phosphoric ester hydrolase activity" and "1-phosphatidylinositol-4-phosphate 5-kinase activity" (Additional file 10: Figure S6). For cell component 10 terms were significantly enriched and "cytosol" was the main supercluster (Additional file 10: Figure S6). The DEGs in cluster 7 were significantly ($p < 0.05$) enriched into 63 GO-terms of biological process, of which the supercluster terms involved "asparagine biosynthesis", "response to stress" and "hemicellulose metabolism" (Fig. 7). Forty-one significant terms for molecular functions were enriched, the superclusters of which involved "xyloglucan:xyloglucosyl transferase activity" and "peroxidase activity" (Additional file 10: Figure S6). Nine significant terms for cell component were enriched, and the supercluster terms of treemap involved "extracellular region" and "external encapsulating structure" (Additional file 10: Figure S6). Genes in the PPI network of cluster 7 were mainly enriched in the GO-terms supercluster "response to stress" and "response to stimulus" (Fig. 6b).

At the same time, we performed KEGG pathway enrichment analysis on the DEGs in the three clusters (Additional file 11: Table S5). DEGs in cluster 5, 6 and 7 were significantly ($p < 0.05$) mapped into 11, 7 and 14 KEGG pathways, respectively. This indicated that there might be more pathways involved in fuzz fiber initiation compared with lint fiber development in high temperature. The most significant pathways in cluster 5 were "Phagosome (14 DEGs)", "Fatty acid elongation (5 DEGs)", "Metabolic pathways (59 DEGs)" and "Amino sugar and nucleotide sugar metabolism (9 DEGs)" (Fig. 5c). The most significant pathways in cluster 6 were "Circadian rhythm (7 DEGs)", "Plant hormone signal transduction (18 DEGs)" and "Brassinosteroid biosynthesis (3 DEGs)" (Fig. 5c). And the top three pathways with the most DEGs in cluster 7 were "Biosynthesis of secondary metabolites (29 DEGs)", "Phenylpropanoid biosynthesis (8 DEGs)" and "Metabolic pathways (32 DEGs)" (Fig. 5c).

Differentially expressed transcription factors involved in fuzz fiber initiation

Numerous studies have shown that the development of *Arabidopsis* trichome or cotton fiber was strictly regulated by several transcription factors [36-42]. To identify the differentially expressed TFs associated with fuzz fiber initiation induced by high-temperature, twenty-five differentially expressed TF genes assigned to thirteen families were detected in cluster 7.

In this study, totally 20 and 105 TF genes were identified in cluster 5 and 6, respectively (Additional file 12: Table S6). Of these, there were more genes involved in lint fiber initiation, and relatively few genes involved in fuzz fiber initiation. Different genes of four families, such as NAC, bHLH, MYB and C2H2, were

found to be participated in lint fiber early development and fuzz fiber initiation in high temperature, respectively. However, transcription factors of four families, such as AP2/ERF(ERF), C2C2-LSD, HD-ZIP and PLATZ, were mainly participated in lint and fuzz fiber initiation. In addition, six genes distributed in five families (*RAV1*, *BLH1*, *HB40*, *LBD1* and *OFP11*) were exclusively enriched in cluster 7. According to the PPI network, *STZ* related to Cys2/His2-type zinc-finger proteins was identified as the key transcription factors of cluster 7, which might play a pivotal role in fuzz fiber initiation induced by high-temperature.

DEGs involved in plant hormone signal transduction pathway

Previous studies have found that cotton fiber development is also regulated by some genes related to plant hormone signal transduction. We identified 189 genes related to hormone signal transduction from 9667 DEGs involved in both temperature response and fiber development. Eighteen unigenes were from cluster 6, including 7 auxin (*AUX1*, *IAA* and *SAUR*), 5 abscisic acid (*PYR/PYL*, *PP2C*, *SnRK2* and *ABF*), 3 cytokinin (*CRE1*, *AHP* and *B-ARR*), 1 ethylene (*CTR1*) and 2 brassinosteroid (*CYCD3*) signal transduction component-encoding genes (Additional file 13: Table S7). Five unigenes were from cluster 7, and these genes encode the components of auxin (*GH3*) and brassinosteroid (*TCH4*) signal transduction, respectively (Additional file 13: Table S7). There were more genes in cluster 6 than that in cluster 7. For example, no genes related to abscisic acid, cytokinin, and ethylene signal transduction were found in cluster 7, but several genes were identified in cluster 6. Although genes for the auxin and brassinosteroid signaling pathways were found in both c6 and c7, the components encoded by these genes were different, indicating that *GH3* and *TCH4* may play a key role in regulating the fuzz fiber initiation induced by high-temperature.

DEGs related to cotton fiber development

According to previous reports, it is believed that the homologous genes related to trichome initiation of *Arabidopsis* in cotton may also be involved in the fiber initiation. We collected more than 100 genes associated with trichome initiation of *Arabidopsis* and identified 695 homologous genes in the *G. barbadense* genome. These 695 genes were compared with 9667 environment-related DEGs, and 56 genes were shared in the two gene sets. Based on the expression profile, we found that some of these genes were affected by temperature (Fig. 8). Among these temperature-responsive genes, the expression of the gene homologous to *ARP3* (*GB_D01G1926* and *GB_A01G1791*), *NAC029* (*GB_A12G1914*), and *MYB82* (*GB_A08G2828*) in L7009 showed an expression trend positively correlated with fuzz fiber initiation development. Hence these genes were identified as candidate genes that might be involved in fuzz fiber initiation induced by high-temperature.

Changes in antioxidant enzyme activity and ROS content during fuzz initiation

According to the GO analysis, some genes, such as *RCI3* (GB_A03G1340), *DOX2* (GB_A09G2023), *PRX52* (GB_A03G0328), *GPX4* (GB_D08G0779) and *ERD10* (GB_A01G1053), potentially related to fuzz initiation were involved in stress response. Therefore, we measured the activities of four antioxidant enzymes and the contents of two ROS (Fig. 9). The results showed that the H₂O₂ content at 4 DPA was significantly higher than 1 or 7 DPA under HT environment, and the content at 4 DPA under LT environment was significantly higher than that of HT environment. The content of OFR in the early development of fibers under HT environment was generally higher than that in LT environment, indicating that the two ROS, OFR and H₂O₂, might play different roles in fuzz fibers initiation development. The activity of the four enzymes showed a downward trend from 1 to 7 DPA under different temperature environments. Among them, the enzyme activities of SOD, CAT and APX at 4 DPA in HT environment were generally higher than those in LT environment. This indicates that the regulation level of ROS homeostasis is an important cause of the difference during fuzz fiber initiation in different temperatures.

Verification of RNA sequencing data by qRT-PCR

To validate the RNA-seq data, quantitative real-time PCR (qRT-PCR) was performed for sixteen genes randomly selected from different expression profiles. The sixteen genes and their primer sequences were listed (Additional file 14: Table S8). In general, the relative expression levels based on qRT-PCR were consistent with the results of RNA-seq and the correlation between them was significant ($R^2=0.78$) (Additional file 15: Figure S7; Fig. 10b). However, when we clustered the sixteen selected genes according to the RNA-seq data and qRT-PCR results, we found that *GB_A01G1791* was not clustered with the other three genes of cluster 7, but with the genes of cluster 2 and 9 (Fig. 10a). We then calculated the correlation between the nine clusters and found that the Pearson correlation of cluster 2 and cluster 9 reached 0.94, indicating that the gene expression patterns from these two clusters are similar (Additional file 16: Table S9). These results indicated that the RNA-seq data was basically credible and accurate.

Discussion

Temperature is the key external factor that determines the fate of epidermal cells of *G. barbadense* ovules and the quality of lint fiber

As an important fiber source plant, *G. barbadense* has been favored by scientific researchers and production industries for its excellent fiber quality traits. The quality of cotton fibers is sensitively affected by environmental stimulus [43, 44]. The difference in fiber quality mainly comes from meteorological factors (e.g. temperature and rainfall) and soil factors [45]. Of these environmental stimulus, temperature is the most important factor affecting fiber quality. Low temperature stress, especially the mean daily minimum temperature (< 20 °C), is the major abiotic stress limiting the formation of cotton fiber quality such as fiber length [46]. In this study, it led to significant differences in fiber quality when the ambient temperature was set to 20~25 °C and 28~36 °C, respectively. Interestingly, high temperature significantly increased the fuzz density (FD) when compared to a low temperature. This indicates that the change of

FD is regulated by the ambient temperature. Moreover, as the FD increased, FL, FS, and FE also increased accordingly. Since FD had a similar change trend with the quality traits of lint fibers, we speculated that the lint fiber quality must be regulated by the ambient temperature, that is, higher temperature will increase FL, FS and FE. However, the underlying mechanism remains unknown and the relevancy between them is noticeable. It is reported that the elevated temperature can indeed accelerate fiber elongation process. Nevertheless, if the temperature is too high, it will shorten the fiber rapid elongation duration and terminate the elongation earlier, leading to the reduction of final fiber length [47]. Similarly, the relative low temperature can also encumber fiber elongation development [48]. Fiber development is often affected by abiotic stresses, such as temperature, salt, drought and waterlogging, and most of them have a negative impact on the formation of fiber quality. We believe that these abiotic stresses may have some commonalities in regulatory pathways and molecular mechanisms in terms of negative regulation of fiber quality.

Utilizing scanning electron microscopy and fiber mutants of *G. hirsutum*, it was found that fuzz fiber initiation occurred at 4 DPA. We also found that the fuzz initiation of *G. barbadense* occurred at 4 DPA. This fully demonstrates that the two allotetraploid cotton species are identical in the fuzz fiber initiation time. Based on the QTL method, some fuzzless locus have been reported, including *N1* and *n2*. The homozygous *N1N1* is completely fuzzless, and the fuzz development of the *n2* mutant is reported to be controlled by genetic background and environmental conditions together [49, 50]. Almost all *G. barbadense* varieties contain the *n2* gene, which makes their fuzz density generally low and is often influenced by the environmental stimulus [51]. A recent study mapped *N1* to chromosome A12, which is thought to be a homologous gene of *MYB25-like* (*GhMML3_A12*) [52]. However, the *n2* gene has still not been discovered, and the environmental stimulus affecting the function of the *n2* gene remain unclear. In this study, we explored the effect of temperature on *G. barbadense* fuzz density and believed that *n2* may also be temperature-regulated.

Major transcription factors involved in fiber development

In cotton, many TF families have been studied, and a large number of TF genes participate in regulating the fiber development and plant responses to environmental factors. In the three clusters, four TF families with the most genes are NAC, bHLH, MYB and GRAS, whose members participate in the development of different types fibers.

NAC transcription factors have multiple functions not only in abiotic stress responses but also in plant development. The role of NAC in plant tolerance against extreme temperature has been demonstrated. In rice, it was reported that *SNAC3* play a positive role in heat resistance through regulating downstream ROS-associated genes expression [53]. In plants, NAC genes overexpression also generated cold tolerance by upregulating some thermotolerance-related genes, as well as inducing the increased activity of antioxidant enzymes [54]. In the present study, 16 genes related to NAC TFs were enriched in the three clusters, and six of these genes were expressed in cluster 7. Of these, *NAC074* was only expressed at 4

DPA in HT but with lower expression at other stages in both high and low temperature. These findings indicate that NAC TFs play a key regulatory role in fuzz fiber initiation in cotton.

The bHLH transcription factors, *AtGL3* and *AtEGL3*, play redundant roles in root hair regulation, and the double mutants have hairy roots [55]. As a bHLH protein, *GhDEL65* can partially restore the trichome development of Arabidopsis *gl3 eg/3* double mutants, and overexpression in wild plants increase the trichome density of leaves and stems [56]. *AtMYC1*, another bHLH gene, functions upstream of *GL2* by interacting with *MYB* and *TTG1* [57]. *GhFP1* (a bHLH gene) as a positive regulator participates in controlling fiber elongation by activating BR biosynthesis and signaling transduction [58]. *GhbHLH18* is negatively associated with fiber strength and length by activating peroxidase-mediated lignin metabolism [59]. In summary, members of the bHLH family not only regulate trichome or fiber initiation, but also control lint fiber elongation and quality. Our results also indicated that bHLH family members were widely involved in the development of lint and fuzz fibers, which further indicates that this TF family members can not only positively regulate lint but also positively regulate fuzz development.

In addition to bHLH and NAC, members of MYB and C2H2 also play an important role in regulating cotton fiber development. As mentioned earlier, the MYB genes play an important role in regulating the initiation and elongation of cotton fibers, such as *GaMYB2*, *GhMYB109*, *GhMYB25*, and *GhMYB25-like* [14, 15]. In this study, two MYB genes homologous with *MYB116* and *MYB305* were expressed in fuzz fiber initiation stages, indicating that they play a role in regulating fuzz development. C2H2 genes have also been reported to play an important role in determining the epidermal cell fate. As members of the C2H2 genes, *GIS*, *ZFP5*, and *ZFP6* promote trichome initiation by transcriptional activation of the downstream trichome initiation complex (GL1-GL3-TTG1) [60]. Our results showed that the only C2H2 gene *STZ* highly expressed during fuzz fiber initiation development. Although few C2H2 genes have been reported to be involved in fiber development in cotton, there should be functional homologous genes of Arabidopsis in cotton according to the regulatory similarity between cotton fibers and *Arabidopsis* trichomes.

Major genes related to phytohormones signal transduction involved in fiber development

The main plant hormones produced by plants include abscisic acid (ABA), auxin, brassinolide (BR), cytokinin (CK), ethylene (ET), gibberellic acid (GA), jasmonates (JA) and salicylic acid (SA). It is well known that many plant hormones are involved in regulating the differentiation and elongation of cotton fiber cells. Brassinolides (BRs) as steroid hormones play an important role in the regulation of plant growth and stress response. Most genes expressed during BR biosynthesis and signal transduction have been reported to be involved in regulation of cotton fiber development. BR biosynthesis begins when campesterol (CR) is catalyzed by DET2 to produce campestanol (CN). DET2, as a steroid 5 α -reductase, is considered to be the rate-limiting enzyme in BR biosynthesis. The loss of this enzyme activity will lead to a reduction in BRs levels [61]. The *GhDET2* is positively regulated during the process of fiber initiation and extreme elongation, and inhibition of this gene will restrain fiber initiation and elongation [62]. *GhPAG1*,

as a homologous gene of *CYP734A1*, controls cotton fibers development by regulating the endogenous BR level through ethylene signal-mediated VLCFA synthesis [63]. *GhBZR1*, as a member of the BR signal transduction cascade, can be regulated by *Gh14-3-3*, and bind to the promoter of *GhXTH1* to regulate fiber initiation and elongation development [64]. As a xyloglucan endotransglycosidase gene, the expression of *TCH4* is induced by auxin, BR, and environmental stimulus and is mainly expressed in trichomes, lateral root primordia, and elongating hypocotyls [65]. In this study, we identified three homologous genes of *TCH4*, which were mainly present in cluster 7 expression pattern. These results indicate that temperature stimulation may control the fuzz initiation development by regulating the expression of genes in BR signal transduction pathways.

In addition, two *GH3* genes related to auxin signal transduction pathway were also identified during fuzz fiber initiation (cluster 7). Studies have found that auxin plays an important role in the initiation and elongation development of cotton fibers [28]. *GhARF2* and *GhARF18* are two homologous genes of *ARF* in the auxin signal transduction pathway, which are mainly expressed during the fiber initiation process [66]. Overexpression of these two genes in *Arabidopsis* promotes the initiation of trichomes. The homologous genes of *IAA*, *GhAux8* and *GhIAA16*, are mainly expressed during the fiber development stage [67]. Of these, *GhAux8* is preferentially expressed in elongating fibers, and *GhIAA16* is expressed in fiber initiation stage. This shows that the IAA signal may play an important role in lint and fuzz fiber initiation stages. This suggests that these two hormones play different roles in the development of the two type fibers, and that the hormones involved in the lint fiber initiation may also be involved in the fuzz fiber initiation.

Maintenance of ROS homeostasis is sufficient to temperature-regulated fiber initiation

Reactive oxygen species (ROS) mainly include superoxide radical, hydrogen peroxide and hydroxyl radical, which can regulate plant cell proliferation and expansion, cell differentiation (such as root hair and lateral root), dedifferentiation and tissue regeneration [68]. Ascorbate peroxidase (APX), a hydrogen peroxide scavenging enzyme, is involved in regulating ROS homeostasis [69]. The expression of *GhAPX1* at 5 DPA in the *fl* mutant ovule was much lower than that of the wild type, and application of H_2O_2 induced *GhAPX1* expression to promote fiber elongation. Enzyme activity and H_2O_2 content assays showed that APX members except *GhAPX1* were mainly involved in the late development of fibers (30 DPA) [70]. *GhPOX1* encodes a class III peroxidase that functions during fiber elongation by mediating ROS production [71]. Studies have shown that homeostasis the H_2O_2 and redox level is the core mechanism leading to inhibition of fiber initiation or elongation [72]. Increased hydrogen peroxide content was detected in 5 DPA ovules of cotton, and H_2O_2 could promote fuzz fiber initiation in vivo [73]. In the present study, we found that a portion of the fuzz fiber initiation-related candidate genes were significantly enriched in the oxidative stress response pathway, in which two hydrogen peroxide scavenging enzyme genes, *GbPrx52* and *GbRCI3*, were found. In *Arabidopsis*, *AtPrx52* is involved in lignin synthesis as a hydrogen peroxide scavenging enzyme, while *AtRCI3* is primarily involved in tolerance

regulation under various abiotic stresses [74, 75]. In this study, the content of H₂O₂ and antioxidant enzymes activity at 4 DPA in the HT environment were lower than that in LT environment, indicating that the ROS level in the HT environment was maintained at a suitable state for the fuzz fiber initiation development, and the ROS homeostasis of LT environment may be disrupted to a certain extent, which may lead to the failure of the fuzz fiber initiation development. In summary, it is believed that the ROS homeostasis regulation must play a key role in fuzz fibers initiation stage.

Conclusions

This study emphasized that thermal stimulus has a significant effect on lint and fuzz fiber development, and validated the regulatory model that temperature determines the fuzz fiber initiation of *G. barbadense*. Low temperature could inhibit the fuzz fiber initiation and delay the lint fiber development, while high temperature could relieve the negative effects of low temperature on these phenotypes. Through comparative transcriptome analysis, a large number of DEGs (9667) involved in both fiber development and temperature response were identified, and three expression patterns closely related to the different development of the fibers were obtained, of which the genes in SC6 were differentially expressed between the two temperature environments during the fuzz fiber initiation. Functional enrichment analysis indicated that DEGs involved in asparagine biosynthetic process, cell wall synthesis and stress response were related to the fuzz fiber initiation induced by high-temperature. Transcription factors, plant hormone signals and ROS homeostasis maintenance may also be potentially involved in fuzz fiber initiation. These findings can better help us understand the morphology and molecular mechanism of fuzz fiber initiation regulated by high temperature.

Materials And Methods

Plant materials and temperature treatments

A total of 121 *G. barbadense* cultivars (provided by the Cotton Research Institute of the Chinese Academy of Agricultural Sciences) were used to investigate the effects of different environment on fuzz density and lint fiber quality. A cultivar named L7009 (provided by Zhejiang Agriculture and Forestry University) was used to evaluate the effects of temperature on fuzz density and fiber quality. We simulated the high temperature environments with 25°C in the day and 20°C at night, and the low temperature environments with 36°C in the day and 28°C at night. Both environments had the same photoperiod (15 h light and 9 h dark) and light intensity. First, we transplanted the three-leaf seedlings into flowerpots with one plant per pot and then transferred them to the greenhouse. When it came to full-bloom stage, 120 cotton plants were equally divided into two groups and transferred into high and low temperature environment, respectively. Dated cards were hanged on the flowers, and the flowers in the HT environment were artificially pollinated everyday. When transferred into the cotton growing rooms on the 15th day, ovules of 0 to 7 DPA in the two environments were separately collected and stored in liquid nitrogen. Each biological replicate at each stage came from a mixed sample of three bolls. The seeds were harvested when the cotton bolls were split and dried. The seeds and lint fibers were manually separated, the fuzz

density was investigated, and the fiber quality was measured. To reveal the time when fuzz fiber initiation occurred, we planted another batch plants of the L7009 cultivar, and the management was the same as the previous batch. To accurately track the temperature changes sensed by each seed during fiber development, we numbered sixty plants in each environment and dated cards were hanged on the flowers every day. After ten days cotton plants from two environments were exchanged. The flowers that bloomed on the day of changing the growing environment were recorded as 0 days after transfer (DT). The flower that bloomed on the day before changing the growing environment was recorded as 1 DT, and flower that bloomed on the first day after changing the growth environment was recorded as -1 DT. Flowers that bloomed every day were tagged with dated cards.

Ovule samples preparation for SEM analysis

Ovule samples collected from HT and LT environments were immersed in electron microscope fixative and vacuumed. The fixed samples were rinsed with 0.1M phosphate buffer PB (PH7.4) for 3 times. The samples were fixed with 1% osmium·0.1M phosphate buffer PB (PH7.4) at room temperature (20°C) for 1~2 h, and then rinsed with 0.1M phosphate buffer PB (PH7.4) for 3 times. The samples were successively dehydrated with 30% to 100% alcohol solution for 15 minutes intervals, and then dehydrated with isoamyl acetate for 15 minutes. After critical point drying and ion sputtering, the sample was observed by scanning electron microscope (SEM).

Fuzz phenotype determination and fibre quality measurement

The fuzz density of seeds was classified into eight grades by visual inspection. The seeds with a score of "0" were fuzzless, and the seeds with a score of "3.5" were covered with dense fuzz fibers. The fuzz density score was used to quantitatively characterize the fuzz phenotype of the seeds, and it was measured in the same way as previously reported [76]. After the fiber and seed were separated manually, the seed size (SS), fuzz density (FD) and the hundred-grain weight (HGW) were determined, respectively. The lint fiber samples were sent to the Center of Cotton Fibre Quality Inspection and Testing, Chinese Ministry of Agriculture (Anyang, Henan province, China) for fiber quality testing, including High Volume Instrument (HVI) analysis and Advanced Fiber Information System (AFIS) analysis.

Determination of physiological and biochemical phenotype

Data analysis showed that some of the genes potentially related to fuzz fiber initiation were involved in stress response and ROS homeostasis, so the activities of SOD, POD, CAT and APX were determined, and the contents of H₂O₂ and oxygen free radical (OFR) were also measured. L7009 ovules were collected from HT and LT environments at three time points based on RNA-seq sampling time, and frozen in liquid nitrogen for later use. The six physiological and biochemical indexes were all determined according to the manufacturer's protocol of assay kits (Comin Biotech, Suzhou, China).

Transcriptome sequencing and data analysis

Ovules of 1, 4, and 7 DPA were collected from HT and LT and conserved in liquid nitrogen, with two biological replicates per treatment. The RNA extraction kit was then used to extract the total RNA according to its standard procedure. RNA purity was measured with a NanoDrop 2000 microspectrophotometer, its concentration was measured with an Agilent 2100 Bioanalyzer, and integrity was monitored with an Agilent RNA 6000 Nano Kit. The cDNA libraries construction and transcriptome sequencing were completed by Annoroad Gene Technology. The company used the PE150 sequencing method of Illumina HiSeq 4000 sequencing platform to complete the sequencing project and feed raw data back to us for further analysis. The clean data were obtained from the raw reads after removing the adaptor sequences and low-quality reads. High-quality sequences were aligned with the latest version genome of *G. barbadense*, which can be available at <http://ibi.zju.edu.cn/cotton>. The FPKM value of each gene in each sample was obtained by Cufflinks software. Genes with FPKM < 0 were removed, and genes with FPKM ≥ 1 in at least one sample were defined as expressed genes. Pearson correlation coefficient analysis and principal component analysis were performed with all FPKM > 0 genes. The edgeR package of R software was used to analyze the differentially expressed genes, and the genes with $|\text{Log}_2\text{FC}| \geq 1$ and $p\text{-value} < 0.01$ were assigned as differentially expressed. The Mfuzz program of R software was used for clustering analysis. GO and KEGG were used for functional annotation and pathway enrichment analysis of genes in important modules. The interaction relationship of genes with membership ≥ 0.70 in each module was obtained from the STRING database (<https://string-db.org/>), and the interaction relationship was imported into Cytoscape to construct a co-expression network diagram.

Validation of RNA-Seq data by qRT-PCR

Total RNA was extracted with RNAprep Pure Plant Plus Kit (Polysaccharides & Polyphenolics-rich). The first strand of cDNA was synthesized using RNA and PrimeScript™ II 1st Strand cDNA Synthesis Kit. All qRT-PCR primers were designed using Oligo7 software and synthesized by Sangon Biotech (Shanghai) Co., Ltd .. The *GbUBQ7* was used as a reference gene. Follow the system and procedures recommended by the UltraSYBR Mixture (Low ROX) instructions. qRT-PCR was performed on an 7500 real-time PCR system. The relative expression of genes was analyzed by $2^{-\Delta\Delta\text{CT}}$ method. The experiment was performed in three biological replicates, with three technical replicates in each biological replicate.

Abbreviations

ABA:Absciscic acid; AFIS:Advanced Fiber Information System; AS:Alternative splicing; BP:Biological process; BR:Brassinosteroid; DAP:Days post anthesis; DEG:Differentially expressed gene; ET:Ethylene; FC:Fold change; FD:Fuzz density; FE:Fiber elongation; FL:Fiber length; FNC:Fiber neps content; FNS:Fiber neps size; FP:Fuzz percentage; FPKM:Fragments per kilobase of exon per million fragments mapped; FS:Fiber strength; GA:Gibberellin; GO:Gene Ontology; HGW:100-grain weight; HT:High temperature; HTL:From high temperature environment to low temperature environment; HVI:High Volume Instrument;

IFC:Immature fiber content; KEGG:Kyoto Encyclopedia of Genes and Genomes; LT:Low temperature; LTH:From low temperature environment to high temperature environment; MR:Mature ratio; PCA:Principal component analysis; PPI:Protein-protein interaction; ROS:Reactive oxygen species; SCNC:Seed coat neps content; SCNMS:Seed coat neps mean size; SFC_n:Short fiber content based on number; SFC_w:Short fiber content based on weight; SS:Seed size; TF:Transcriptional factor; UQL:Upper quartile length

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data included in this article and the additional files are available. The transcriptome datasets of *G. barbadense* cultivar L7009 are deposited in the NCBI database with a SRA accession number SUB6753336.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by the China Agriculture Research System (CARS-15-06) and the Chinese National Natural Science Foundation (grant No. 31601346).

Authors' contributions

GMC and SXY conceived and designed this study. GMC wrote the manuscript. GMC and LYZ performed most of the experiments. GMC and LYZ analyzed the RNA-seq data. HLW, HTW and JHL revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We are grateful to Junkang Rong (a professor affiliated to Zhejiang A&F University) for providing us with *G. barbadense* cultivar L7009 and cotton growing greenhouses.

References

1. Zhang W, Huang L, Li J, Li Y, Wei S, Cai L, Wu H: **Transcriptomic analysis of differentially expressed genes in the oviduct of *Rhacophorus omeimontis* provides insights into foam nest construction.** *BMC Genomics* 2019, **20**(1):562.
2. Qin YM, Zhu YX: **How cotton fibers elongate: a tale of linear cell-growth mode.** *Curr Opin Plant Biol* 2011, **14**(1):106-111.
3. Graves DA, Stewart JM: **Chronology of the differentiation of cotton (*Gossypium hirsutum* L.) fiber cells.** *Planta*, **175**(2):254-258.
4. Lee JJ, Hassan OS, Gao W, Wei NE, Kohel RJ, Chen XY, Payton P, Sze SH, Stelly DM, Chen ZJ: **Developmental and gene expression analyses of a cotton naked seed mutant.** *Planta* 2006, **223**(3):418-432.
5. Zhang D-Y, Zhang T-Z, Sang Z-Q, Guo W-Z: **Comparative Development of Lint and Fuzz Using Different Cotton Fiber-specific Developmental Mutants in *Gossypium hirsutum*.** *Journal of Integrative Plant Biology* 2007, **49**(7):1038-1046.
6. Snowden MC, Ritchie GL, Simao FR, Bordovsky JP: **Timing of Episodic Drought Can Be Critical in Cotton.** *Agronomy Journal*, **106**(2).
7. Pettigrew, T. W: **The Effect of Higher Temperatures on Cotton Lint Yield Production and Fiber Quality.** *Crop Science*, **48**(1):278.
8. Zhou M, Sun G, Sun Z, Tang Y, Wu Y: **Cotton proteomics for deciphering the mechanism of environment stress response and fiber development.** *Journal of Proteomics*, **105**:74-84.
9. Chen J, Burke JJ: **Developing fiber specific promoter-reporter transgenic lines to study the effect of abiotic stresses on fiber development in cotton.** *PLoS One* 2015, **10**(6):e0129870.
10. Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A: **The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci.** *Development*, **135**(11):1991-1999.
11. Gan Y, Liu C, Yu H, Broun P: **Integration of cytokinin and gibberellin signalling by *Arabidopsis* transcription factors GIS, ZFP8 and GIS2 in the regulation of epidermal cell fate.** *Development* 2007, **134**(11):2073-2081.
12. Zhou Z, An L, Sun L, Gan Y: **ZFP5 encodes a functionally equivalent GIS protein to control trichome initiation.** *Plant Signal Behav* 2012, **7**(1):28-30.
13. Zhou Z, Sun L, Zhao Y, An L, Yan A, Meng X, Gan Y: **Zinc Finger Protein 6 (ZFP6) regulates trichome initiation by integrating gibberellin and cytokinin signaling in *Arabidopsis thaliana*.** *New Phytol* 2013, **198**(3):699-708.
14. Walford SA, Wu Y, Llewellyn DJ, Dennis ES: **GhMYB25-like: a key factor in early cotton fibre development.** *Plant J* 2011, **65**(5):785-797.
15. Machado A, Wu Y, Yang Y, Llewellyn DJ, Dennis ES: **The MYB transcription factor GhMYB25 regulates early fibre and trichome development.** *Plant J* 2009, **59**(1):52-62.

16. Pu L, Li Q, Fan X, Yang W, Xue Y: **The R2R3 MYB transcription factor GhMYB109 is required for cotton fiber development.** *Genetics* 2008, **180**(2):811-820.
17. Shan CM, Shangguan XX, Zhao B, Zhang XF, Chao LM, Yang CQ, Wang LJ, Zhu HY, Zeng YD, Guo WZ *et al*: **Control of cotton fibre elongation by a homeodomain transcription factor GhHOX3.** *Nat Commun* 2014, **5**:5519.
18. Walford SA, Wu Y, Llewellyn DJ, Dennis ES: **Epidermal cell differentiation in cotton mediated by the homeodomain leucine zipper gene, GhHD-1.** *Plant J* 2012, **71**(3):464-478.
19. Deng F, Tu L, Tan J, Li Y, Nie Y, Zhang X: **GbPDF1 is involved in cotton fiber initiation via the core cis-element HDZIP2ATATHB2.** *Plant Physiol* 2012, **158**(2):890-904.
20. Cho HT, Cosgrove DJ: **Regulation of root hair initiation and expansin gene expression in Arabidopsis.** *Plant Cell* 2002, **14**(12):3237-3253.
21. De Grauwe L, Vandenbussche F, Tietz O, Palme K, Van Der Straeten D: **Auxin, ethylene and brassinosteroids: tripartite control of growth in the Arabidopsis hypocotyl.** *Plant Cell Physiol* 2005, **46**(6):827-836.
22. Sun Y, Veerabomma S, Abdel-Mageed HA, Fokar M, Asami T, Yoshida S, Allen RD: **Brassinosteroid regulates fiber development on cultured cotton ovules.** *Plant Cell Physiol* 2005, **46**(8):1384-1391.
23. Shi YH, Zhu SW, Mao XZ, Feng JX, Qin YM, Zhang L, Cheng J, Wei LP, Wang ZY, Zhu YX: **Transcriptome profiling, molecular biological, and physiological studies reveal a major role for ethylene in cotton fiber cell elongation.** *Plant Cell* 2006, **18**(3):651-664.
24. Lee JJ, Woodward AW, Chen ZJ: **Gene expression changes and early events in cotton fibre development.** *Ann Bot* 2007, **100**(7):1391-1401.
25. Traw MB, Bergelson J: **Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in Arabidopsis.** *Plant Physiol* 2003, **133**(3):1367-1375.
26. Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K: **Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in Arabidopsis.** *Development* 2009, **136**(6):1039-1048.
27. Wang L, Zhu Y, Hu W, Zhang X, Cai C, Guo W: **Comparative Transcriptomics Reveals Jasmonic Acid-Associated Metabolism Related to Cotton Fiber Initiation.** *PLoS One* 2015, **10**(6):e0129854.
28. Xiao G, Zhao P, Zhang Y: **A Pivotal Role of Hormones in Regulating Cotton Fiber Development.** *Front Plant Sci* 2019, **10**:87.
29. Liu Z, Liu SQ, Xiao-Nanb YU, Chen JX: **Response of fertile pollen grains to different temperatures and cytological base of male sterility in TemianS-1.** *Journal of Hunan Agricultural University* 2007.
30. Chen R, Zhao X, Shao Z, Wei Z, Wang Y, Zhu L, Zhao J, Sun M, He R, He G: **Rice UDP-Glucose Pyrophosphorylase1 Is Essential for Pollen Callose Deposition and Its Cosuppression Results in a New Type of Thermosensitive Genic Male Sterility.** *Plant Cell*, **19**(3):847-861.
31. Bokszczanin KL, Solanaceae Pollen Thermotolerance Initial Training Network C, Fragkostefanakis S: **Perspectives on deciphering mechanisms underlying plant heat stress response and thermotolerance.** *Front Plant Sci* 2013, **4**:315.

32. Sakata T: **Auxins reverse plant male sterility caused by high temperatures.** *Plant Signaling & Behavior* 2010, **107**(11):8569-8574.
33. Chhun T, Aya K, Asano K, Yamamoto E, Morinaka Y, Watanabe M, Kitano H, Ashikari M, Matsuoka M, Ueguchi-Tanaka M: **Gibberellin Regulates Pollen Viability and Pollen Tube Growth in Rice.** *Plant Cell*, **19**(12):3876-3888.
34. YL S, YH J, SP H, ZL Z, JL S, BY P, XM D: **Genetic analysis of fuzzless in cotton germplasm.** 2012, **34**(8):1073.
35. Supek, Bošnjak F, Škunca M, Šmuc N, Tomislav: **REVIGO summarizes and visualizes long lists of Gene Ontology terms.** *Plos One* 2011, **6**(7):e21800.
36. Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A: **The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci.** *Development* 2008, **135**(11):1991-1999.
37. Matias-Hernandez L, Aguilar-Jaramillo AE, Cigliano RA, Sanseverino W, Pelaz S: **Flowering and trichome development share hormonal and transcription factor regulation.** *J Exp Bot* 2016, **67**(5):1209-1219.
38. Zhou L, Zheng K, Wang X, Tian H, Wang X, Wang S: **Control of trichome formation in Arabidopsis by poplar single-repeat R3 MYB transcription factors.** *Front Plant Sci* 2014, **5**:262.
39. Tominaga-Wada R, Nukumizu Y, Sato S, Wada T: **Control of plant trichome and root-hair development by a tomato (*Solanum lycopersicum*) R3 MYB transcription factor.** *PLoS One* 2013, **8**(1):e54019.
40. Zhao H, Li X, Ma L: **Basic helix-loop-helix transcription factors and epidermal cell fate determination in Arabidopsis.** *Plant Signal Behav* 2012, **7**(12):1556-1560.
41. Mo H, Wang L, Ma S, Yu D, Lu L, Yang Z, Yang Z, Li F: **Transcriptome profiling of *Gossypium arboreum* during fiber initiation and the genome-wide identification of trihelix transcription factors.** *Gene* 2019, **709**:36-47.
42. Bedon F, Ziolkowski L, Walford SA, Dennis ES, Llewellyn DJ: **Members of the MYBMIXTA-like transcription factors may orchestrate the initiation of fiber development in cotton seeds.** *Frontiers in Plant Science* 2014, **5**.
43. Davidonis GH, Johnson AS, Landivar JA, Fernandez CJ: **Cotton Fiber Quality is Related to Boll Location and Planting Date.** 2004, **96**(1):42-47.
44. Campbell BT, Jones MA: **Assessment of genotype × environment interactions for yield and fiber quality in cotton performance trials.** **144**(1-2):69-78.
45. Zhang Z, Zhu L, Song A, Wang H, Chen S, Jiang J, Chen F: **Chrysanthemum (*Chrysanthemum morifolium*) CmlCE2 conferred freezing tolerance in Arabidopsis.** *Plant Physiol Biochem* 2020, **146**:31-41.
46. Zheng M, Wang Y, Liu K, Shu H, Zhou Z: **Protein expression changes during cotton fiber elongation in response to low temperature stress.** *Journal of Plant Physiology*, **169**(4):0-409.

47. Dai Y, Yang J, Hu W, Zahoor R, Chen B, Zhao W, Meng Y, Zhou Z: **Simulative Global Warming Negatively Affects Cotton Fiber Length through Shortening Fiber Rapid Elongation Duration.** *Sci Rep* 2017, **7**(1):9264.
48. Wuzi X, Trolinder NL, Haigler CH: **Cool Temperature Effects on Cotton Fiber Initiation and Elongation Clarified Using In Vitro Cultures.** *Crop Science*, **33**(6).
49. Turley RB, Kloth RH: **Identification of a Third Fuzzless Seed Locus in Upland Cotton (*Gossypium hirsutum* L.).** *Journal of Heredity* 2002, **93**(5):359-364.
50. Turley RB, Vaughn KC, Scheffler JA: **Lint development and properties of fifteen fuzzless seed lines of Upland cotton (*Gossypium hirsutum* L.).** *Euphytica*, **156**(1-2):57-65.
51. Zhu QH, Yuan Y, Stiller W, Jia Y, Wang P, Pan Z, Du X, Llewellyn D, Wilson I: **Genetic dissection of the fuzzless seed trait in *Gossypium barbadense*.** *Journal of Experimental Botany*, **69**(5):997-1009.
52. Wan Q, Guan XY, Yang NN, Wu HT, Pan MQ, Liu BL, Fang L, Yang SP, Hu Y, Ye WX *et al.* **Small interfering RNAs from bidirectional transcripts of GhMML3_A12 regulate cotton fiber development.** *New Phytologist* 2016, **210**(4).
53. Fang Y, Liao K, Du H, Xu Y, Song H, Li X, Xiong L: **A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice.** *J Exp Bot* 2015, **66**(21):6803-6817.
54. Marques DN, Reis SP, Souza CR: **Plant NAC transcription factors responsive to abiotic stresses.** *Plant Gene* 2017, **11**:170-179.
55. Bernhardt, C.: **The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root.** *Development*, **130**(26):6431-6439.
56. Shangguan XX, Yang CQ, Zhang XF, Wang LJ: **Functional characterization of a basic helix-loop-helix (bHLH) transcription factor GhDEL65 from cotton (*Gossypium hirsutum*).** *Physiol Plant* 2016, **158**(2):200-212.
57. Zhao H, Wang X, Zhu D, Cui S, Li X, Cao Y, Ma L: **A single amino acid substitution in IIIf subfamily of basic helix-loop-helix transcription factor AtMYC1 leads to trichome and root hair patterning defects by abolishing its interaction with partner proteins in Arabidopsis.** *J Biol Chem* 2012, **287**(17):14109-14121.
58. Liu ZH, Chen Y, Wang NN, Chen YH, Wei N, Lu R, Li Y, Li XB: **A basic helix-loop-helix (bHLH) protein (GhFP1) promotes fiber elongation of cotton (*Gossypium hirsutum*) via modulating BR biosynthesis and signaling.** *New Phytol* 2019.
59. Gao Z, Sun W, Wang J, Zhao C, Zuo K: **GhbHLH18 negatively regulates fiber strength and length by enhancing lignin biosynthesis in cotton fibers.** *Plant Sci* 2019, **286**:7-16.
60. Chang J, Yu T, Yang Q, Li C, Xiong C, Gao S, Xie Q, Zheng F, Li H, Tian Z *et al.* **Hair, encoding a single C2H2 zinc-finger protein, regulates multicellular trichome formation in tomato.** *Plant J* 2018, **96**(1):90-102.
61. Fujioka, S.: **The Arabidopsis deetiolated2 Mutant Is Blocked Early in Brassinosteroid Biosynthesis.** *Plant Cell*, **9**(11):1951-1962.

62. Luo M, Xiao Y, Li X, Lu X, Deng W, Li D, Hou L, Hu M, Li Y, Pei Y: **GhDET2, a steroid 5 α -reductase, plays an important role in cotton fiber cell initiation and elongation.** *Plant J* 2007, **51**(3):419-430.
63. Yang Z, Zhang C, Yang X, Liu K, Wu Z, Zhang X, Zheng W, Xun Q, Liu C, Lu L *et al*: **PAG1, a cotton brassinosteroid catabolism gene, modulates fiber elongation.** *New Phytol* 2014, **203**(2):437-448.
64. Ying, Zhou, Ze-Ting, Zhang, Mo, Li, Xin-Zheng, Wei, Xiao-Jie, Li: **Cotton (*Gossypium hirsutum*) 14-3-3 proteins participate in regulation of fibre initiation and elongation by modulating brassinosteroid signalling.**
65. Xu SR, Wei P, Yang QL, Jia GX, Ma SK, Yang QE, Jun Z, Zhang RN: **Transcriptome analysis revealed key signaling networks regulating ovarian activities in the domestic yak.** *Theriogenology* 2020, **147**:50-56.
66. Xiao G, He P, Zhao P, Liu H, Yu J: **Genome-wide Identification of GhARF gene Family Reveals GhARF2 and GhARF18 are Involved in Cotton Fibre Cells Initiation.** *Journal of Experimental Botany* 2018, **69**(18).
67. Han X, Xu X, Fang DD, Zhang T, Guo W: **Cloning and expression analysis of novel Aux/IAA family genes in *Gossypium hirsutum*.** *Gene* 2012, **503**(1):83-91.
68. Schmidt R, Schippers JH: **ROS-mediated redox signaling during cell differentiation in plants.** *Biochim Biophys Acta* 2015, **1850**(8):1497-1508.
69. Li HB, Qin YM, Pang Y, Song WQ, Mei WQ, Zhu YX: **A cotton ascorbate peroxidase is involved in hydrogen peroxide homeostasis during fibre cell development.** *New Phytol* 2007, **175**(3):462-471.
70. Tao C, Jin X, Zhu L, Xie Q, Wang X, Li H: **Genome-wide investigation and expression profiling of APX gene family in *Gossypium hirsutum* provide new insights in redox homeostasis maintenance during different fiber development stages.** *Mol Genet Genomics* 2018, **293**(3):685-697.
71. Mei W, Qin Y, Song W, Li J, Zhu Y: **Cotton GhPOX1 encoding plant class III peroxidase may be responsible for the high level of reactive oxygen species production that is related to cotton fiber elongation.** *Journal of Genetics and Genomics* 2009, **36**(3):141-150.
72. Guo K, Du X, Tu L, Tang W, Wang P, Wang M, Liu Z, Zhang X: **Fibre elongation requires normal redox homeostasis modulated by cytosolic ascorbate peroxidase in cotton (*Gossypium hirsutum*).** *J Exp Bot* 2016, **67**(11):3289-3301.
73. Du SJ, Dong CJ, Zhang B, Lai TF, Du XM, Liu JY: **Comparative proteomic analysis reveals differentially expressed proteins correlated with fuzz fiber initiation in diploid cotton (*Gossypium arboreum* L.).** *J Proteomics* 2013, **82**:113-129.
74. Fernandez-Perez F, Pomar F, Pedreno MA, Novo-Uzal E: **The suppression of AtPrx52 affects fibers but not xylem lignification in *Arabidopsis* by altering the proportion of syringyl units.** *Physiol Plant* 2015, **154**(3):395-406.
75. Llorente F, López-Cobollo RM, Catalá R, Martínez-Zapater JM, Salinas J: **A novel cold-inducible gene from *Arabidopsis*, RCI3, encodes a peroxidase that constitutes a component for stress tolerance.** *Plant Journal* 2002, **32**(1):13-24.

76. Zhu QH, Yuan Y, Stiller W, Jia Y, Wang P, Pan Z, Du X, Llewellyn D, Wilson I: **Genetic dissection of the fuzzless seed trait in *Gossypium barbadense***. *J Exp Bot* 2018, **69**(5):997-1009.

Supplementary Files Legend

Additional file 1: Figure S1. Visual grading of fuzz density.

Additional file 2: Figure S2. Correlation between fiber quality and fuzz density. The correlation coefficient is given by r -value, and the correlation significance is marked by p -value. The data came from 121 samples of island cotton planted in Sanya, Hainan province in 2016. The fiber quality was measured by High Volume Instrument (HVI), and the fuzz density was graded by manual visual measurement.

Additional file 3: Table S1. The p -value matrix for the comparisons between the same and different treatments.

Additional file 4: Figure S3. Cotton fiber quality developed in development temperature environments measured by AFIS and HVI. Twelve fiber quality traits measured by Advanced Fiber Information System (AFIS): Length_w (length based on weight), Length_n (length based on number), SFC_w (short fiber content based on weight), SFC_n (short fiber content based on number), UQL (upper quartile length), Fineness, MR (mature ratio), IFC (Immature fiber content), FNC (fiber neps content), FNS (fiber neps size), SCNC (seed coat neps content), and SCNMS (seed coat neps mean size). The fiber quality trait of Uniformity and FE (fiber elongation) measured by HVI. Three seed traits, such as HGW (100-grain weight), SS (seed size), and FP (fuzz percentage), were determined by artificial methods. The significant difference was represented by p -value.

Additional file 5: Table S2. Alternative splicing prediction and novel genes annotation.

Additional file 6: Table S3. All expressed genes with FPKM value ≥ 1 .

Additional file 7: Figure S4. Venn diagram of differentially expressed genes (DEGs) from low temperature (LT), high temperature (HT) and the comparison between different temperatures (HL). The overlapping parts of the different ellipses represent the number of DEGs common to these comparison groups. The gene sets labelled with orange number represent genes that involved in both temperature response and fiber development.

Additional file 8: Figure S5. Clusters of expression patterns of DEGs involved in both temperature response and fiber development. The change of expression level is shown after Z-score conversion. The Mfuzz program of R software was used for clustering analysis.

Additional file 9: Table S4. The enriched GO terms of the three clusters.

Additional file 10: Figure S6. Treemap of cellular component and molecular function GO-terms found in three clusters. In each treemap, rectangles represent significant GO-terms. GO-terms sharing the same

color belong to the same GO superclusters whose names are labelled in white color. The sizes of rectangles reflect the relative corrected p -value (the larger the rectangle, the more significantly the GO-term was).

Additional file 11: Table S5. KEGG enrichment of genes in the three clusters.

Additional file 12: Table S6. Differentially expressed transcription factors of three clusters.

Additional file 13: Table S7. DEGs involved in plant hormone signal transduction pathway in the three clusters.

Additional file 14: Table S8. Primers for qRT-PCR

Additional file 15: Figure S7. The relative gene expression of sixteen randomly selected genes examined by qRT-PCR. Correlation coefficients between RNA-seq data and qRT-PCR expression data were correspondingly represented by r -values. All values were shown with mean $\pm$ SE.

Additional file 16: Table S9. Pearson correlation between the nine Mfuzz clusters.

Figures

Figure 1

Dynamic change of fuzz density and verification of environmental factor. (a) The change of fuzz density between the three natural environments. Fuzz density with seven levels and percentages of corresponding cultivars are shown. The width of the strip indicates the number of varieties. (b) Statistics of daily minimum temperature during the three-year flowering period in the three natural environments. Daily minimum temperature is divided into two groups of 24~36°C and 15~22°C, which represent the number of high temperature days and low temperature days, respectively. (c) Real-time temperature monitoring of artificial climate chamber. (d) Fuzz density in HT and LT environments. The number of seeds investigated at HT and LT was $n = 64$ and $n = 57$, respectively. The number of asterisks represents a significant level (***, $p < 0.001$).

Figure 2

Validation the time of fuzz fiber initiation. After being moved from HT to LT, the dynamic variation of fuzz density with time (a), and the significant difference of fuzz density between different treatment depths (b). After being moved from LT to HT, the dynamic variation of fuzz density with time (c), and the significant difference (p-value) matrix of fuzz density between different treatment depths (d).

Figure 3

Impact evaluation of different temperatures on developing ovules, fuzz density and fiber quality. (a) Scanning electron microscopy of ovules and corresponding epidermal fibers affected by high and low temperatures during fuzz fiber development. Scale bar: 500 μm (the first two columns of the first row and the third row); 1 mm (the third column of the first row); 200 μm (the second and fourth rows). Rows two and four are enlarged versions of the SEM images on rows one and three, respectively. (b) Mature seeds developed in high (Temp > 28°C, n = 191) and low (Temp < 25°C, n = 311) temperature environments. (c) The fiber quality of fiber length (FL), fiber strength (FS) and Micronaire developed under high and low temperature environments measured by HVI system.

Figure 4

Relationship between transcriptome samples and loop comparison of DEGs within and between HT and LT. (a) Cluster dendrogram and correlation coefficient heatmap based on normalized FPKM values of expressed genes. (b) PCA analysis of transcriptome data from 12 samples. The dots in the bisque background represent samples from the HT environment, and the dots in the powderblue background represent samples from the LT environment. (c) Loop comparisons of the differentially expressed genes within and between the two environments at three stages during fuzz fiber development. Samples are represented by stage numbers with environmental prefixes, such as H1 and L1, which represent ovules of 1 DPA in HT and LT, respectively. The red numbers indicate the up-regulated genes, and the blue numbers indicate the down-regulated genes. (d) Venn diagram of up-regulated DEGs (upper) and down-regulated DEGs (lower) within and between HT and LT at different stages or at the same stages.

Figure 5

Hierarchical cluster analysis of DEGs. (a) Heatmap of the expression profiles of 9667 DEGs participating in both temperature response and fiber development. The color scale represents the Z-score of the gene expression level. (b) Trend analysis of genes in the three clusters related to fiber and fuzz development. (c) KEGG pathway enrichment analysis of the three clusters related to fiber development. The color scale on the left of the heatmap represents different levels of significance.

Figure 6

PPI network analysis of the genes in specific module. (a) PPI network based on co-expressed genes in cluster 7. The larger the circle, the more genes that interact with it in the module. The circles with gradient colors represent the hub genes, and their names are highlighted in bold. (b) Treemap of BP GO-terms found in the PPI network. Each rectangle represents a single cluster of related terms. Related GO terms are clustered together in superclusters of the same color. The size of each rectangle is adjusted to reflect the p-value of the enrichment of the GO terms.

Figure 7

Treemap of BP GO-terms found in the three clusters. Each rectangle represent a significant GO-term. Related GO terms are clustered together in superclusters of the same color and supercluster names are labelled in white. The sizes of rectangles are adjusted to reflect the relative corrected p-value.

Figure 8

Heatmap of the genes potentially related to fiber development.

Figure 9

Antioxidant enzyme activities and ROS levels under different temperature environments during early fiber development. The height of the bar represents the mean value, and the error bar represents mean ± SE.

Figure 10

Validation of the RNA-seq data by qRT-PCR. (a) Heat map of RNA-seq data and qRT-PCR data of 16 selected genes from three clusters. (b) Correlation between RNA-seq data (FPKM) and qRT-PCR results (2- $\Delta\Delta$ Ct) of 16 selected genes. Scatter points represent the fold-changes in expression levels at 4 DPA compared with 1 DPA. The R² value represents the correlation between RNA-seq data and qRT-PCR results.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS4.xlsx
- TableS3.xlsx
- FigureS1.tif
- TableS9.xlsx
- FigureS6.tif
- FigureS7.tif
- FigureS4.tif
- FigureS5.tif
- TableS8.xlsx
- TableS7.xlsx
- FigureS3.tif
- TableS5.xlsx
- TableS6.xlsx
- FigureS2.tif
- TableS2.xlsx
- TableS1.xlsx