

Transcriptome Analysis of Ten-DPA Fiber in an Upland Cotton (*Gossypium hirsutum*) Line with Improved Fiber Traits from Phytochrome A1 RNAi Plants

Qing Miao¹, Peng Deng², Sukumar Saha³, Johnie N. Jenkins³, Chuan-Yu Hsu⁴,
Ibrokhim Y. Abdurakhmonov⁵, Zabardast T. Buriev⁵, Alan Pepper⁶, Din-Pow Ma^{1*}

¹Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS, USA

²Department of Pharmacology, Weill Cornell Medical College, New York, NY, USA

³USDA-ARS, Crop Science Research Laboratory, Mississippi State, MS, USA

⁴Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University, Mississippi State, MS, USA

⁵Center of Genomics and Bioinformatics, Academy of Sciences of Uzbekistan, Tashkent, Uzbekistan

⁶Department of Biology, Texas A & M University, College Station, TX, USA

Email: *dma@bch.msstate.edu

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Abstract

Silencing phytochrome A1 gene (*PHYA1*) by RNA interference in Upland cotton (*Gossypium hirsutum* L. cv. Coker 312) had generated *PHYA1* RNAi lines with improved fiber quality (longer, stronger and finer fiber). To reveal molecular mechanisms that govern fiber development with positive fiber traits, a study of global gene expression profiling of 10-DPA fibers in a *PHYA1* RNAi line and its parent Coker 312 was conducted by high-throughput RNA sequencing. A comparative analysis of transcriptomes between the two lines had identified 142 genes that were differentially expressed in the 10-DPA fiber of the RNAi line. Gene Ontology analysis showed that these differentially expressed genes were mainly involved in metabolic pathways, heterocyclic/organic cyclic compound binding and multiple enzyme activities, and cell structures which were reported to play important roles in fiber development. Twenty-eight KEGG pathways were mapped for the 142 genes, and the pathways related to glycolysis/gluconeogenesis and pyruvate metabolism were the most abundant and followed by cytochrome P450-involved pathways, suggesting that fiber improvement could be through the regulation of proteins involved in cytochrome P450 pathways. Genes encoding WRKY transcription factors, sucrose synthase, xyloglucan endotransglucosylase hydrolase, udp-glucuronate: xy-lan alpha-glucuronosyltransferase, and genes involved in lipid metabolism and

ABA/brassinosteroid signal transduction pathways were found differentially expressed in the RNAi line. These genes have direct impacts on cotton fiber quality. The results of this study elucidate molecular signatures and possible mechanisms of fiber improvement in the background of *PHYA1* RNAi in cotton and should help for future fine-tuning and programming of cotton fiber development.

Keywords

Fiber Development, Phytochrome A1, RNA Interference, RNA-Seq, Transcriptome

1. Introduction

Cotton is the most important textile fiber and also an important oilseed crop [1], providing significant economic impact as a major cash crop in 70 counties around the world [2]. The cotton industry demands to produce cotton with good fiber qualities, including longer and finer fiber with high fiber strength. Most of these desirable fiber traits are controlled by many genes (QTLs) and are very difficult to improve simultaneously by conventional breeding program because some of these traits are inversely related. Cotton fiber is a single cell derived from the epidermal layer of the ovule of a cotton seed, and its development consists of four overlapping stages: initiation, elongation (primary wall biosynthesis), secondary wall biosynthesis and maturation [3]. The elongation stage encompasses the major expansion growth phase of the fiber, which may last for 25 to 30 days post anthesis (DPA) [4]. During elongation, fiber deposits a thin, expandable primary cell wall composed of a variety of carbohydrate polymers [5]. As the fiber approaches the end of elongation, the major phase of secondary wall synthesis starts [3].

Previous studies showed that silencing phytochrome A1 gene (*PHYA1*) by RNA interference generated RNAi cotton lines with improved fiber quality (longer, stronger and finer fiber) and some other phenotypic changes, including early-flowering, vigorous root and vegetative growth, without adverse effects on yield and fiber development, and higher tolerance to abiotic stresses when compared to non-transformed Coker 312 [6] [7]. Phytochromes are red and far-red light photoreceptors, and a high ratio of far-red light to red light perceived by the photoreceptors increased fiber length [8]. Phytochromes were reported to mediate plant hormone signaling through direct interaction between their negative regulators phytochrome-interacting factors (PIFs) and certain phytohormone signaling components, such as DELLA proteins [9]. Phytohormones play roles in mediating cotton fiber development. Auxins and gibberellins (GA3) promote early stages of fiber initiation and have a positive effect on fiber elongation [10]. Abscissic acid (ABA) inhibits fiber initiation by counteracting the promotion of auxins and gibberellins [10]. Ethylene (ET) plays an important role

in promoting fiber cell elongation [10]. In addition, ET- and brassinosteroid (BR)-related genes are up-regulated during fiber elongation [11]. Over the past decades, many genes have been reported to be involved in cotton fiber development. Genes encoding MYB transcription factors play a role in fiber cell fate determination and development [12]. Fiber cells in elongation stage are dynamically changed both in components and structures. Many genes involved in carbohydrate metabolism and transportation are up-regulated during fiber development [12]. The development of fiber cells also depends on higher turgor pressure, and enzymes and proteins for maintaining high turgor pressure are enriched in growing fiber cells [12]. In addition, wall-loosening proteins also play roles in fiber elongation [12]. Recently, genome-wide analyses of gene expression via comparative transcriptome profiling have identified many genes that are differentially expressed in fiber cells at the four developmental stages [13] [14].

In this study, high-throughput RNA sequencing, a powerful tool, was used to uncover how silencing the *PHYA1* gene could lead to the improvement of fiber quality and agricultural traits in the RNAi line. A total of 142 genes were found to be differentially expressed in *PHYA1* RNAi cotton compared to Coker 312. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis suggested that improved fiber quality of RNAi cotton might be due to the regulation of some critical transcription factors and genes involved in carbohydrate metabolism and phytohormone signaling. Cytochrome P450 involved pathways and other differentially expressed genes (DEGs) might play a crucial role in fiber and abiotic tolerance improvement of RNAi plants. The identified and characterized genes that were differentially expressed in *PHYA1* RNAi cotton lines provided useful information for future fine-tuning and better genetic improvement of cotton.

2. Materials and Methods

2.1. Plant Materials and Plant Growth

With the aim to analyze the function of phytochrome A1 gene (*PHYA1*) of cotton in association with fiber length [15], a binary RNAi construct was previously developed by PCR amplification of the 213 bp hinge region of *PHYA1* gene from *G. hirsutum* TM-1 genomic DNA and cloned into the PHellsgate-8 vector using the Gateway Cloning System (Thermo Fisher Scientific) [6]. The RNAi construct was then somatically transformed into embryogenic *Gossypium hirsutum* L. cv. Coker 312 to generate transgenic cotton plants [6]. The resulting *PHYA1*-specific single-seed decent RNAi transgenic plants were subsequently self-pollinated through first to sixth generations (T1-7-6) and followed by selection of individual plants in each generation for phytochrome-associated traits (including plant architecture, hypocotyl length, vigorous growth, early flowering, superior fiber quality, etc.) and genomic insertions of the RNAi hairpin construct. The RNAi plants of T2-6 generations were then grown in field stations at Tashkent, Uzbekistan.

kistan under normal solar light conditions and evaluated for agronomic performance and stability of the RNAi effects [6] [7]. The seeds of single seed decent T1-7 family RNAi plants of the sixth generation were transferred to the USDA-ARS laboratory at Mississippi State, Mississippi, USA to evaluate RNAi plant performance under the USA environments. Comparative phenotypic evaluations of US-grown RNAi and Coker 312 plants were conducted for various morphological and agronomic traits including fiber quality properties.

Seeds of individually selected PCR-positive (containing the RNAi construct) T6-7 RNAi homozygous plants were grown in the field at the North Farm R. R. Foil Plant Science Research Center of Mississippi State University. Flowers were tagged on the day of anthesis (0 DPA). Cotton bolls (10 DPA) were harvested with three biological replications on August 10, 2015 from 9 - 10 am. Fibers were carefully dissected from cotton bolls, immediately frozen in liquid nitrogen, and then stored at -80°C for RNA extraction.

2.2. RNA-Seq Library Construction and Illumina Sequencing

Total RNA was isolated from 10 DPA fibers using a modified hot borate method [16]. Genomic DNA contamination in the RNA samples was removed with DNase I (Promega, Madison, WI, USA). Briefly, 10 - 50 μg of total RNA were treated with 5 units of DNase I at 37°C for 20 min in a 50 μl reaction according to the manufacturer's instructions. After DNase treatment, the RNA sample was cleaned up with a Qiagen RNasey Mini Kit (Qiagen, Valencia, CA, USA). The quality of the RNA sample was pre-checked on a 1% - 1.2% agarose gel by electrophoresis, and the RNA concentration was determined with Nanodrop 2000c and a Qubit® fluorometer by using a Qubit RNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). The quality of total RNA was finally estimated using the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

Two μg of total RNA with three biological replicates per genotype were used to construct RNA-Seq libraries using the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. All 6 RNA-Seq libraries were independently barcoded with 6 compatible TruSeq RNA indexes (Illumina, San Diego, CA, USA) for multiplexing. The size of each library was analyzed with Bioanalyzer DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA), and the concentration of each library was quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and qPCR assays. Based on the results from qPCR assays, all libraries were diluted to the concentration of 20 nM and further pooled together with equal volume from each library. The pooled library sample was sequentially subjected to HiSeq 2500 system with pair-ended 50 bp (2×50) sequencing (Illumina, San Diego, CA, USA) for the transcriptomic analysis.

2.3. Data Processing and Analysis

FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to

test data quality. Trimmomatic [17] was used to trim the low-quality reads and adaptor sequences. *Gossypium hirsutum* L. acc. TM-1 genome [18] was used as reference for short reads mapping. ArrayStar (Version 12, DNASTAR Inc., Madison, WI, USA) was used for RNA-Seq analysis. The expression of genes was normalized by the Reads Per Kilobase of transcript per Million mapped reads (RPKM) method [19]. Blast2GO [20] and InterProScan 5 [21] were used for identifying orthologous genes and adding additional GO terms, if necessary. GO terms were mapped to the GO-slim [22] to get a broad functional overview of the transcriptome. The Fisher's exact test method [23] was used for gene set enrichment analysis [24] [25]. KEGG database [26] was used for additional protein function analysis. The mRNA sequencing data of Coker 312 and *PHYA1* line were deposited into NCBI with accession numbers SRP099141 and SRP099143, respectively.

2.4. Quantitative RT-PCR

Total RNA of 10-DPA fiber was used as template for first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The qRT-PCR reaction was carried out on lightcycler 480 (Roche, Basel, Switzerland) with the following cycling profile: 95°C for 5 min, following by 45 cycles of 10 sec at 95°C, 15 sec at 60°C and 15 sec at 72°C. Each qRT-PCR reaction mixture contained 10 µl of SYBR Green I Master (Roche, Basel, Switzerland), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 2 µl of cDNA (4 × fold dilution), and 6 µl of ddH₂O. The transcript of cotton Ubiquitin 7 gene (GhUBQ7) was used to normalize the expression levels of RT-PCR products [27]. All reactions were performed with three replicates. The $2^{-\Delta\Delta Ct}$ method [$\Delta Ct = Ct$ (differentially expressed gene) – Ct (UBQ7), $\Delta\Delta Ct = \Delta Ct$ (RNAi) – ΔCt (Coker 312), $2^{-\Delta\Delta Ct}$ = Relative Expression] was used to calculate relative expression of differentially expressed genes [28]. All primers were designed by using the IDT online tool (<https://www.idtdna.com/scitools/Applications/RealTimePCR/>).

3. Results

3.1. Sequence Statistics of Six 10-DPA Fiber Libraries

The preliminary results from 2015-2016 USA field evaluations in replicated plots suggested significant differences in fiber properties between the RNAi and Coker 312. The RNAi line in average had fiber length of 1.21 inch and strength of 31.8 gm/tex versus Coker 312 control with fiber length of 1.16 inch and strength of 29.4 gm/tex (Jenkins *et al.* 2017; unpublished results). To determine global transcriptome differences in elongated fibers between *PHYA1* RNAi and Coker 312 lines, the 10-DPA fiber RNA samples from three individual plants of each line were used to generate 6 independent libraries that were subsequently sequenced using the Illumina HiSeq 2500 sequencing platform. Approximately 565.8 million reads of 50-bp long were generated from all 6 libraries (Table 1). Low quality

Table 1. Total reads statistics of RNA-Seq libraries.

Sample	Coker 312			RNAi		
	CF 1	CF 2	CF 3	RF 1	RF 2	RF 3
Total Sample Reads	94,720,967	93,523,005	94,121,986	95,064,738	94,462,679	93,860,621
Total Reads Mapped	94,694,073	92,987,578	93,840,825	94,718,530	94,205,949	93,693,368
Total Unque Reads	28,996,162	27,493,684	28,244,922	28,662,405	27,759,879	26,857,354

Note: CF and RF denote the fibers from Coker 312 and *PHYA1* RNAi plants, respectively. The RNA samples from Coker 312 and *PHYA1* RNAi lines have 3 biological replicates (replicates 1 - 3).

reads and adaptor sequences were then trimmed off with Trimmomatic [17] and the clean reads were mapped to 70,478 transcripts from the *Gossypium hirsutum* L. acc. TM-1 genome sequence [18].

3.2. Global Transcriptome Changes in *PHYA1* RNAi Plant Fiber Development

The mapped sequenced reads of all identified transcripts were used for differential expression analysis with the ArrayStar program with a FDR less than 0.05. The overall expression patterns of the mapped genes between the RNAi line and Coker 312 were similar with a linear correlation $R^2 = 0.9747$ (Figure 1). A total of 142 genes (fold change ≥ 2) were either up or down regulated in *PHYA1* RNAi plant compared to Coker 312. Of these genes, 138 genes were down-regulated and 4 genes were up-regulated. The 142 genes differentially expressed in *PHYA1* 10-DPA fibers were listed in Table 2. RNA-Seq data showed high levels of transcripts of *PHYA1* sequence tags in RNAi line compared to wild-type Coker 312, which was due to the transcription of the hinge region (213 bp) of the *PHYA1* gene in the RNAi hairpin construct under the strong constitutive CaMV 35S promoter. Because extremely low counts of RNA-seq tags (below 10) were mapped to the *PHYA1* coding region after filtering data from the hinge region, it was not feasible to use statistical tools to determine whether there was a difference in *PHYA1* expression between RNAi and Coker 312 lines. Our qRT-PCR results also indicated that *PHYA1* was expressed at very low levels in fiber, and no significant difference in *PHYA1* transcript levels was observed between the two lines in 10-DPA fibers (data not shown).

The transcript levels of many transcription factor families were significantly changed. For example, 11 genes in the WRKY gene family (Gh_D08G1232, Gh_A08G2417, Gh_D04G1318, Gh_A03G0210, Gh_D05G0600, Gh_D06G1078, Gh_D03G1371, Gh_A06G1923, Gh_A05G0483, Gh_A06G0917 and Gh_A07G0-017) encoding WRKY transcription factors or like proteins were down-regulated in 10-DPA fibers of the *PHYA1* RNAi line compared to Coker 312. The WRKY gene family had been revealed to participate in multiple fiber developmental processes [29]. Two genes encoding ABA 8'-hydroxylase (Gh_A08G1344 and Gh_D08G1639), a type of cytochrome P450 monooxygenase, in catalyzing the first step in oxidative degradation of ABA were down-regulated [30] [31]. ABA

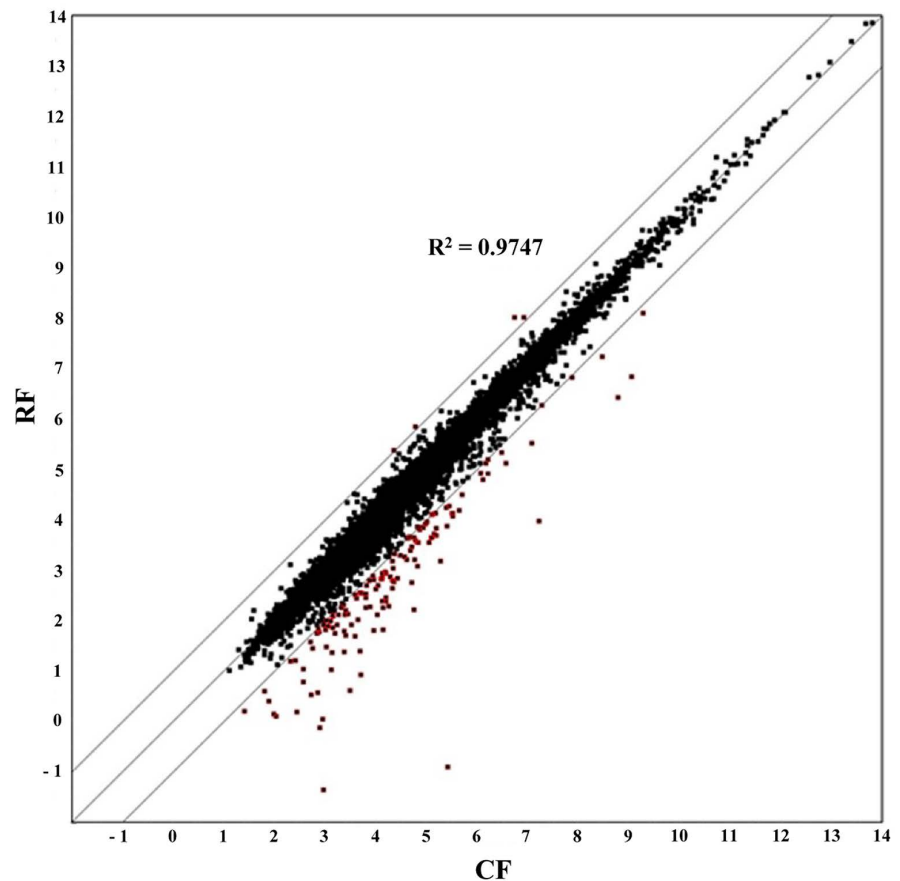


Figure 1. Transcriptome scatter plot of *PHYA1* RNAi plant (RF) compared to Coker 312 (CF). Red data points indicate genes that show ≥ 2 -fold changes in expression with a FDR adjusted $p \leq 0.05$, $R^2 = 0.9747$.

was reported to play a negative role in fiber development [11]. Certain proteins and enzymes which are involved in cell wall biogenesis and organization process were also affected by silencing the *PHYA1* gene. For example, the expression of 4 genes encoding xyloglucan endotransglucosylase hydrolase family protein (Gh_D02G0992, Gh_A05G2724, Gh_D05G3030 and Gh_A05G3992) and the gene Gh_A11G0519 for udp-glucuronate:xylan alpha-glucuronosyltransferase 1-like, a cell wall-modifying enzyme, were down-regulated. In addition, sucrose synthases (Gh_A08G1031 and Gh_D08G1309) and proteins including linoleate 13s-lipoxygenase (Gh_A10G0504) and non-specific lipid transfer protein (Gh_A09G1902) related to lipid metabolism were also affected.

These differentially expressed genes (DEGs) were distributed across all 26 chromosomes in the tetraploid progenitor A-genome and D-genome (Figure 2). Of these 142 genes, 68 genes were originated from A-genome, and 71 genes were from D-genome. In A-genome, the DEGs were most abundantly located in chromosome 5 with 12 genes, which had 18% of the DEGs distributed in A-genome. Following the chromosome 5 was the chromosome 8 with 10 DEGs, composed of 15% of the group. For D-genome, similar distribution patterns were found; chromosomes 19 and 24 had the highest numbers of DEGs, but chromosome

Table 2. The list of 142 differentially expressed genes (fold change ≥ 2) in *PHYA1* RNAi line.

Gene Accession Number (NBI)	Gene Annotation	Fold Change
Gh_D01G0906	phosphoprotein ecpp 44-like	0.0125
Gh_D08G1232	probable wrky transcription factor 41	0.05
Gh_A05G1317	pyruvate decarboxylase 1	0.1048
Gh_D10G2595	---NA---	0.1233
Gh_A08G2417	probable wrky transcription factor 41	0.133
Gh_D04G1318	probable wrky transcription factor 33	0.1359
Gh_D02G0992	xyloglucan endotransglucosylase hydrolase family protein	0.1463
Gh_D04G1245	bahd acyltransferase dcr	0.1735
Gh_D02G0733	alcohol dehydrogenase	0.1948
Gh_D07G1319	uncharacterized calcium-binding protein atlg02270-like isoform $\times 1$	0.1988
Gh_D08G1639	abscisic acid 8-hydroxylase 1-like	0.2031
Gh_D12G1525	lov domain-containing protein	0.2059
Gh_D05G3509	scarecrow-like protein 21	0.2105
Gh_A02G0689	alcohol dehydrogenase	0.2159
Gh_A05G0931	probable protein phosphatase 2 c 25	0.218
Gh_D08G2557	phosphatase 2 c family isoform 1	0.2244
Gh_A08G1344	abscisic acid 8-hydroxylase 1-like	0.233
Gh_D02G0732	alcohol dehydrogenase class-p-like	0.2338
Gh_D09G0953	probable ccr4-associated factor 1 homolog 11	0.2504
Gh_D05G0148	ein3-binding f-box protein 1-like	0.2542
Gh_D08G2556	nuclease harbi1	0.2573
Gh_A05G0839	exocyst complex component exo70b1-like	0.2622
Gh_A08G2389	oxalate--ligase	0.265
Gh_D04G0293	nac domain-containing protein 2-like	0.2686
Gh_A09G0923	probable ccr4-associated factor 1 homolog 11	0.271
Gh_A05G2138	serine threonine-protein kinase-like protein ccr4	0.28
Gh_D06G1403	ethylene-responsive transcription factor 4-like	0.2846
Gh_D07G0283	PREDICTED: uncharacterized protein LOC105769700	0.2886
Gh_A10G0504	linoleate 13s-lipoxygenase 3-chloroplastic	0.2941
Gh_A08G0965	nadp-specific glutamate	0.2982
Gh_D05G3030	xyloglucan endotransglucosylase hydrolase family protein	0.299

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Gh_D06G1513	endoplasmic reticulum oxidoreductin-1-like	0.3001
Gh_D08G2484	ein3-binding f-box protein 1-like	0.3076
Gh_D02G1480	probable ribose-5-phosphate isomerase 2	0.31
Gh_A05G2724	xyloglucan endotransglucosylase hydrolase family protein	0.3247
Gh_A08G2191	nuclease harbi1	0.3272
Gh_D07G2014	nam protein	0.33
Gh_A08G2112	ein3-binding f-box protein 1-like	0.3311
Gh_A07G1811	nam protein	0.3327
Gh_D01G1679	---NA---	0.3346
Gh_D05G1208	tetratricopeptide repeat-like superfamily	0.3389
Gh_D08G1346	protein rolling stone-like isoform x2	0.3394
Gh_D03G1189	scarecrow-like protein 34	0.3401
Gh_A03G0210	probable wrky transcription factor 25	0.3409
Gh_A13G1709	nuclease harbi1	0.346
Gh_D01G1033	zinc finger protein zat10-like	0.3467
Gh_D10G2455	zinc finger ccch domain-containing protein 29-like	0.3469
Gh_A03G2061	protein yls9-like	0.3497
Gh_D05G0600	wrky transcription factor 40-like protein	0.3524
Gh_A03G0889	phosphate transporter pho1 homolog 3-like	0.3552
Gh_D08G0665	auxin-induced protein 22 b	0.3636
Gh_D12G0236	maternal effect embryo arrest 14 isoform 1	0.3643
Gh_A10G1443	protein srg 1	0.3655
Gh_D01G0450	ethylene-responsive transcription factor 4-like	0.3663
Gh_D02G0832	ethylene-responsive transcription factor rap2-4-like	0.3667
Gh_A09G1902	non-specific lipid-transfer protein at2g13820	0.3691
Gh_D02G0417	ethylene receptor 2	0.3707
Gh_A03G2168	---NA---	0.3749
Gh_Sca006021G02	calcium-dependent lipid-binding family	0.3758
Gh_A04G0305	ethylene-responsive transcription factor 4-like	0.381
Gh_A08G1031	sucrose synthase 1	0.3839
Gh_A06G1144	ethylene-responsive transcription factor 4-like	0.389
Gh_A10G2134	zinc finger ccch domain-containing protein 29-like	0.39
Gh_D06G1078	wrky transcription factor 40-like protein	0.3922

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Gh_D03G1371	probable wrky transcription factor 25	0.3947
Gh_A02G0671	u-box domain-containing protein 28-like	0.3974
Gh_D12G0679	maternal effect embryo arrest isoform partial	0.4007
Gh_A07G0226	hypothetical protein F383_30923	0.4008
Gh_A12G2296	heavy metal-associated domain protein	0.4038
Gh_A11G1993	probable indole-3-acetic acid-amido synthetase	0.4044
Gh_A01G0875	prolyl endopeptidase	0.4047
Gh_D08G2140	probable protein phosphatase 2 c 25	0.4066
Gh_A12G0132	nitronate monooxygenase	0.4074
Gh_A10G0255	ethylene-responsive transcription factor 4-like	0.408
Gh_D02G1817	---NA---	0.4085
Gh_D06G0817	ethylene-responsive transcription factor erf017-like	0.4092
Gh_A05G0434	ein3-binding f-box protein 1	0.4098
Gh_A06G1910	nac domain-containing protein 2	0.4106
Gh_D03G1188	scarecrow-like protein 30	0.411
Gh_A03G1618	probable protein phosphatase 2 c 63	0.4134
Gh_A05G3992	xyloglucan endotransglucosylase hydrolase family protein	0.4146
Gh_A06G1923	probable wrky transcription factor 40	0.4189
Gh_D08G1949	4-coumarate--ligase-like 10	0.423
Gh_A09G0534	stem-specific protein tsjt1	0.4249
Gh_A05G0483	wrky transcription factor 40 -like protein	0.4298
Gh_Sca013634G01	two-component response regulator-like aprr5 isoform x2	0.4309
Gh_D05G3348	ethylene-responsive transcription factor 4-like	0.4321
Gh_D11G3494	disease resistance protein rga4 isoform x1	0.4323
Gh_A12G1612	disease resistance protein rga3	0.4323
Gh_A05G2211	respiratory burst oxidase homolog protein d-like	0.4323
Gh_A11G0519	udp-glucuronate: xylan alpha-glucuronosyltransferase 1-like	0.436
Gh_D02G2032	probable protein phosphatase 2 c 63	0.4368
Gh_A01G1439	expansin-like a2	0.4384
Gh_D10G1768	acetate butyrate--ligase peroxisomal-like	0.4392
Gh_A03G1070	probable ribose-5-phosphate isomerase 2	0.4397
Gh_A01G1726	protein yls9-like	0.4404
Gh_D07G1307	suppressor of disruption of tfiis	0.4435

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Gh_D13G0440	classical arabinogalactan protein 9	0.4437
Gh_A04G0196	scarecrow-like protein 21	0.4467
Gh_A06G0917	wrky transcription factor 40 -like protein	0.4499
Gh_D04G1573	glutamyl-trna amidotransferase subunit a	0.4514
Gh_A05G1814	lob domain-containing protein 39-like	0.4515
Gh_A05G3754	wat1-related protein at4g08300-like	0.452
Gh_A08G1791	probable protein phosphatase 2 c 25	0.4529
Gh_D03G1808	late embryogenesis abundant hydroxyproline-rich glycoprotein isoform 1	0.4549
Gh_A13G1311	9-cis-epoxycarotenoid dioxygenase chloroplastic-like	0.4563
Gh_D02G0837	protein exordium-like	0.4565
Gh_D06G1046	ein3-binding f-box protein 1-like	0.4572
Gh_A05G3796	cyclin family isoform 1	0.4592
Gh_A11G0944	endonuclease or glycosyl hydrolase with c2h2-type zinc finger isoform 1	0.4615
Gh_D05G0562	ein3-binding f-box protein 1-like	0.4656
Gh_A07G1599	probable sodium-coupled neutral amino acid transporter 6	0.4673
Gh_D06G2235	scarecrow-like protein 13	0.4675
Gh_A11G0020	purple acid phosphatase 17-like	0.4688
Gh_A06G0709	ethylene-responsive transcription factor erf017-like	0.472
Gh_D05G1276	inorganic pyrophosphatase 1-like	0.475
Gh_D11G3015	soybean gene regulated by cold-	0.4753
Gh_D12G2716	two-component response regulator-like aprr5 isoform x1	0.4763
Gh_D02G1271	phosphate transporter pho1 homolog 3-like	0.4765
Gh_A10G1517	acetate butyrate--ligase peroxisomal-like	0.4783
Gh_D05G2829	gata transcription factor 8-like	0.4803
Gh_A09G0414	scarecrow-like protein 8	0.4814
Gh_A10G2086	uncharacterized acetyltransferase at3g50280-like	0.4815
Gh_A07G0017	probable wrky transcription factor 17	0.4832
Gh_A08G0581	calcium-transporting atpase plasma membrane-type	0.4834
Gh_A03G0639	udp-glycosyltransferase 89b1-like	0.4841
Gh_A08G1509	homeobox-leucine zipper protein hat4-like	0.4847
Gh_A10G2166	senescence associated gene	0.4847
Gh_A02G1410	phosphoenolpyruvate carboxykinase	0.4873
Gh_D11G1089	endonuclease or glycosyl hydrolase with c2h2-type zinc finger isoform 1	0.4874

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Gh_D04G0606	e3 ubiquitin-protein ligase ring1-like	0.4914
Gh_A06G1742	scarecrow-like protein 13	0.4954
Gh_D01G2344	cytochrome P450 87a3-like	0.4956
Gh_D08G1309	sucrose synthase 1	0.4972
Gh_D09G1773	nac domain-containing 78 -like protein	0.4977
Gh_A01G1640	calmodulin-binding protein 25-like	0.4985
Gh_D10G0255	ethylene-responsive transcription factor 4-like	0.4985
Gh_D10G0773	glycerophosphodiester phosphodiesterase gde1-like	0.4989
Gh_D11G2932	pectinesterase inhibitor-like	2.054
Gh_D13G0889	cytosolic sulfotransferase 12-like	2.112
Gh_Sca016160G01	uncharacterised protein	2.1153
Gh_D05G0805	pollen ole e 1 allergen and extensin familyable copy ^a	2.4296

NA: not annotated.

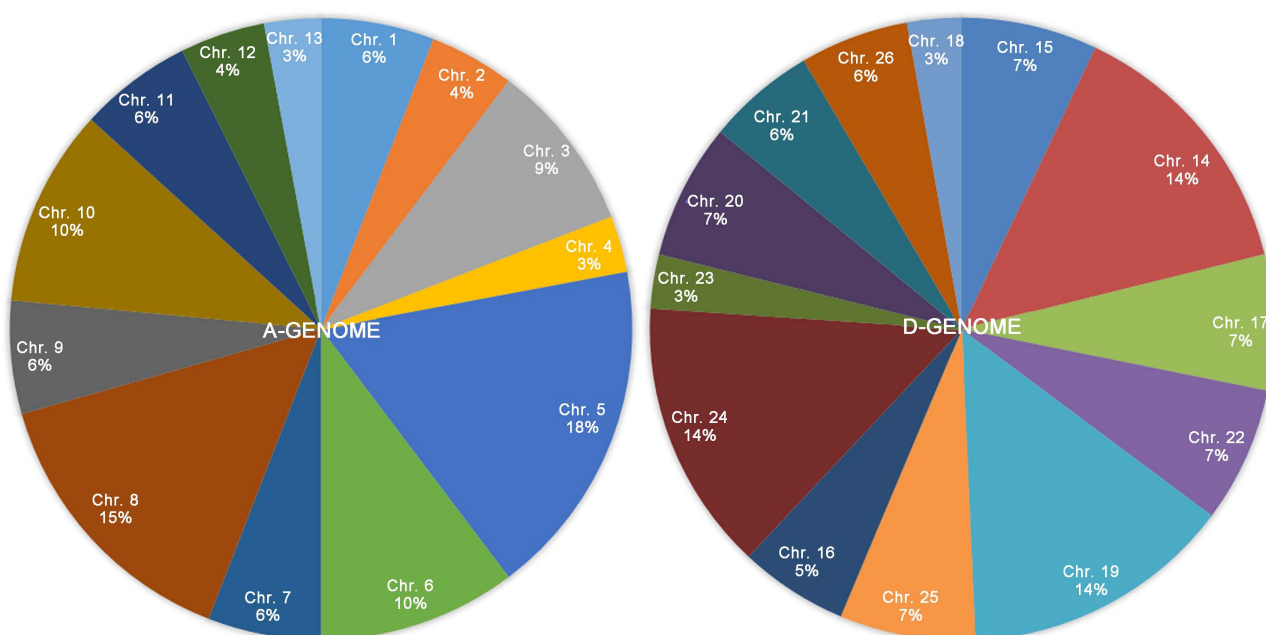


Figure 2. Chromosomal distribution of 142 differentially expressed genes. Distribution of differentially expressed transcripts was determined by the alignment of *PHYA1* RNAi RNA-seq reads to the draft genome of the allotetraploid *Gossypium hirsutum* L. acc. TM-1.

14 also had identical numbers of DEGs as chromosomes 19 and 24. All the three chromosomes 19, 24 and 14 had 10 DEGs, each having 14% DEGs in D-genome (Figure 2).

3.3. Gene Annotation of DEGs

The 142 differentially expressed genes were annotated by Blast2GO [20], and additional GO terms were added to DEGs by InterProScan 5 [21]. All the DEGs

were subjected to GO enrichment analysis and grouped into three subsections: molecular function, biological process, and cellular components by Blast2GO (Figure 3). The results from the enrichment analysis indicated that most of the identified DEGs were involved in cellular metabolic process, heterocyclic/organic cyclic compound binding and multiple enzyme (hydrolase, transferase, and oxidoreductase) activities, and cell structures which have been reported to play important roles in fiber development (Figure 3).

3.4. KEGG Pathway Analysis

To identify metabolic pathways in the RNAi line during the fiber elongation stage, the 142 DEGs were mapped to KEGG database [26]. As a result, 28 pathways were mapped in the database and they were mostly related to glycolysis/gluconeogenesis, pyruvate metabolism, and metabolism by cytochrome P450 (Figure 4).

3.5. Validation of RNA Sequencing Results by Quantitative Real-Time PCR Analysis (qRT-PCR)

To test whether the RNA-seq gene expression results were reliable, RNA transcripts of 15 DEGs in *PHYA1* RNAi plant were selected for qRT-PCR analysis

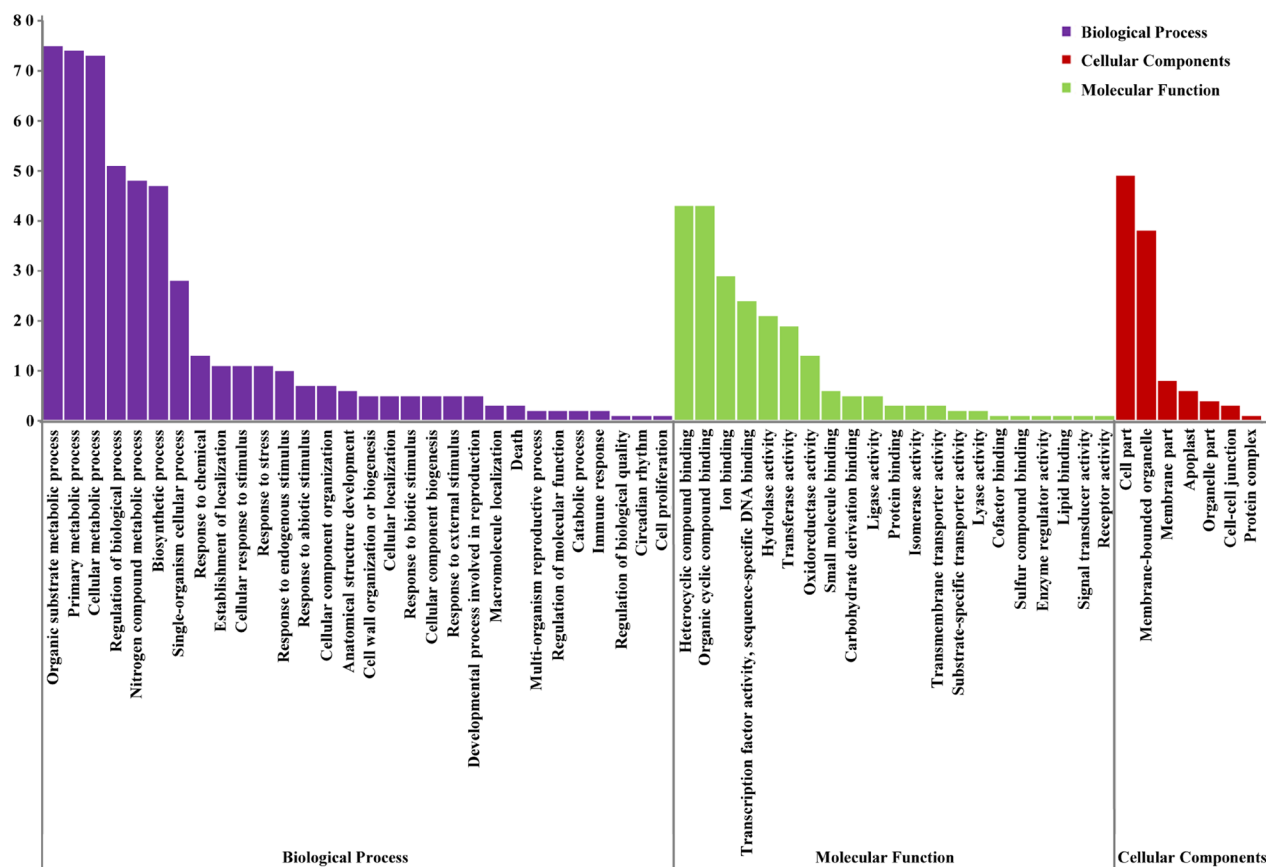


Figure 3. Gene ontology categories of differentially expressed genes (fold change ≥ 2) in *PHYA1* RNAi line compared to Coker 312. The number of differentially expressed genes in each category is shown in the vertical axis.

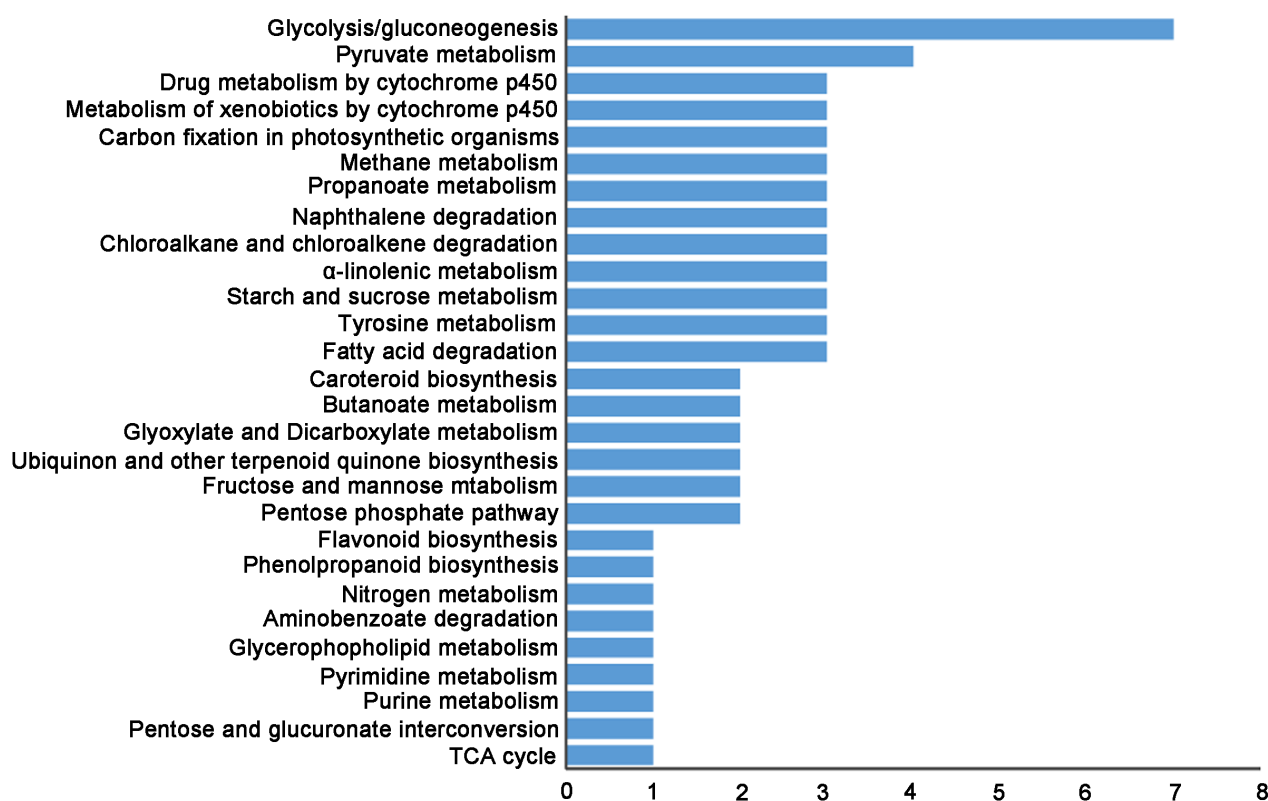


Figure 4. Kyoto encyclopedia of genes and genomes pathway classifications of differentially expressed genes. The number of differentially expressed transcripts in each pathway is shown.

(primers used for qRT-PCR are listed in [Table 3](#)). A comparison of the expression of each transcript by qRT-PCR and RNA-seq was made and shown in [Figure 5](#). All the 15 transcripts were successfully detected by qRT-PCR, and the expression patterns of these transcripts showed general agreement with RNA-Seq results. These 15 transcripts include four up-regulated genes ([Figure 5](#)). Three out of the four genes were successfully annotated and they were pollen ole e1 allergen and extension family protein (Gh_D05G0805), cytosolic sulfotransferase 12-like protein (Gh_D13G0889), and pectinesterase inhibitor-like protein (Gh_D11G2932). The gene with the accession number Gh_Sca016160G01 was uncharacterized ([Table 2](#)). The rest of the 11 genes were down-regulated, and four of them had more than 8-fold down-regulation in RNAi plants. The expression levels of three out of the four genes were consistent with the results from RNA-seq, and they encoded WRKY transcription factor 41 (Gh_D08G1232), pyruvate decarboxylase 1 (Gh_A05G1317), and an unknown gene (Gh_D10G2595). A phosphoprotein ecpp44-like protein (Gh_D01G0906) gene was about 80-fold down-regulated in RNAi plants via RNA sequencing, however, it only showed approximately 4-fold down-regulation by the qRT-PCR method ([Table 2](#) and [Figure 5](#)). This discrepancy between RNA-seq and qRT-PCR results could have been caused by very low counts of transcripts detected in the RNAi line, which were usually unreliable for down-stream analysis. The other seven genes include Gh_D09G0953 (probable ccr4-associated factor 1 homolog 11), Gh_D05G3509

Table 3. Primers used for quantitative RT-PCR.

Primer name	Primer sequences (5'→3')
Gh_D01G0906F	GCGAGGGATGTTTGATTCTTG
Gh_D01G0906R	CCAGGAGACTGTGTTCTTCTC
Gh_D08G1232F	TGTGCCAGAGTCTCCTCTAA
Gh_D08G1232R	CCACCTAGGCATCATCTTTCTC
Gh_A05G1317F	GAAAGCAAGCCCGTTTACATTAG
Gh_A05G1317R	TACCTTTGGAGCGAGAAAGAAG
Gh_D10G2595F	CCATAACCGCGACCTTAAC
Gh_D10G2595R	CGGCAACTTCTTCACTGTTTC
Gh_D02G0992F	TCGAGTTCCTCGGGAATTTAAG
Gh_D02G0992R	CAGTTGGGTCAAACCAGAGATA
Gh_D02G0733F	GCAGAAGGATGAAGTCCGTATTA
Gh_D02G0733R	GACCTAGTATGCGAGGAAACAG
Gh_D08G1639F	GGTGTCCCTGTGTGATGATT
Gh_D08G1639R	TGCCCCAACATCCTCTCTTTAC
Gh_D05G3509F	GAGGTCCTTATGTGCGGATTAC
Gh_D05G3509R	CTGCTCGGATAGTTCCATCAAA
Gh_A05G0931F	TCCGTCCGGGTCTCTATTATC
Gh_A05G0931R	TTGGCCTCTTCTCTCAATATC
Gh_D09G0953F	GAGGAGGCAAGGGATTGATTT
Gh_D09G0953R	CCCAACTCACCGACTCATTAC
Gh_D05G0148F	CTCCTGTGCTTGTGTCTCTAAG
Gh_D05G0148R	CGTTCTCCTTGACCACCTTT
Gh_D11G2932F	CTTTGGCACTTCACGAACAAG
Gh_D11G2932R	AACTCACGCCTCTGGTAAAC
Gh_D13G0889F	CTTTGGCACTTCACGAACAAG
Gh_D13G0889R	AACTCACGCCTCTGGTAAAC
Gh_Sca016160G01F	AGTGCTTTACAACCCGAAGG
Gh_Sca016160G01R	ACACGGTCCAGACTCCTAC
Gh_D05G0805F	GTGGACCAAACCAGCTATACA
Gh_D05G0805R	AGGGCATGAAGTGGAGAAAG
Gh_UBQ7F	GAAGGCATTCCACCTGACCAAC
Gh_UBQ7R	CTTGACCTTCTTCTTCTGTGCTTG

(scarecrow-like protein 21), Gh_D05G0148 (ein3-binding f-box protein 1-like), Gh_D08G1639 (abscisic acid 8'-hydroxylase 1-like), Gh_A05G0931 (probable protein phosphatase 2c 25), Gh_D02G0733 (alcohol dehydrogenase), and Gh_D02G0992 (xyloglucan endotransglucosylase hydrolase family protein) were all down-regulated in the RNAi line, which were consistent with the results

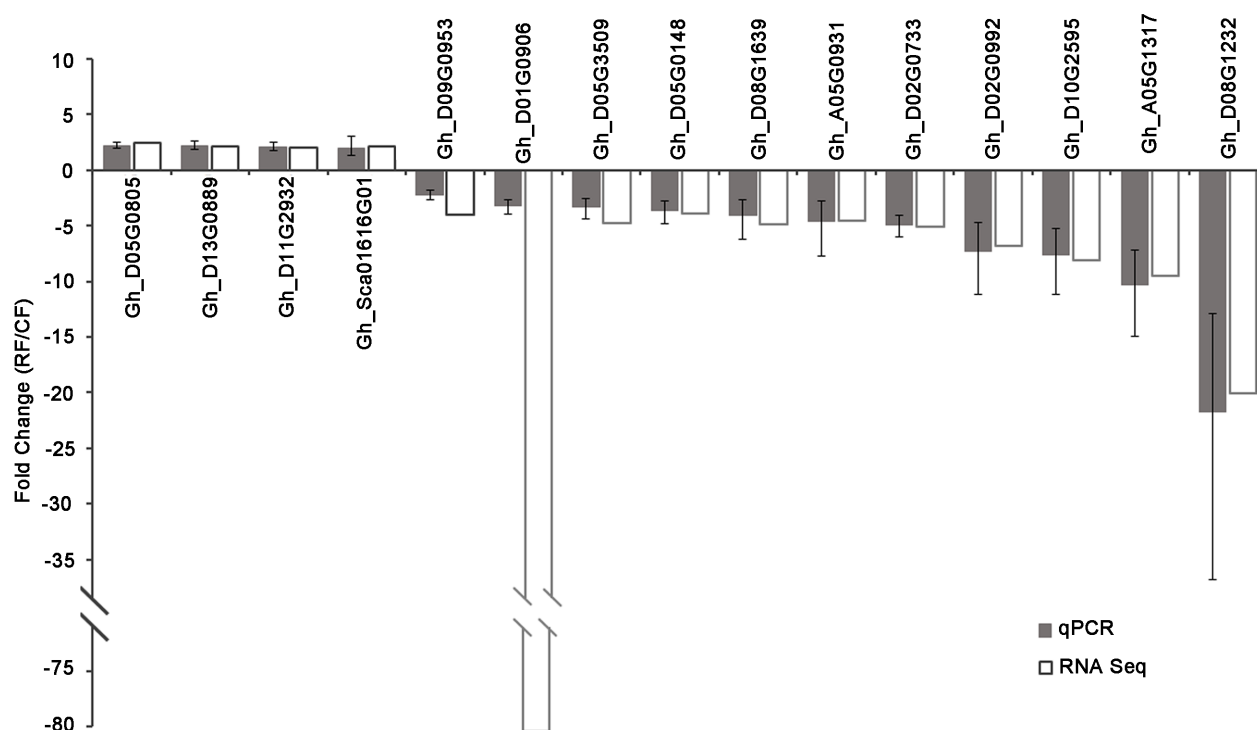


Figure 5. Validation of the expression patterns of differentially expressed genes in 10 DPA fiber of *PHYA1* RNAi line by qRT-PCR. Gene expression was represented as relative fold change $2^{-\Delta\Delta C_T}$ ($\Delta\Delta C_T = \Delta C_T \text{ RNAi} - \Delta C_T \text{ Coker 312}$). The expression levels of genes were normalized by using GhUBQ7 as a reference. Three biological replicates and three technical replicates were included for qRT-PCR experiments. The error bar represents the confidence limits.

from RNA-Seq (Table 2 and Figure 5). In summary, the majority (except for Gh_D01G0906) of the selected transcripts tested by qRT-PCR showed similar expression patterns as determined by the statistical analysis of RNA-Seq data.

4. Discussion

4.1. Transcriptome Profiling of 10-DPA Fibers in RNAi and Coker 312 Lines

The *PHYA1* RNAi cotton was reported to have a 5% increase in fiber length compared to non-transformed Coker 312 [6]. The field evaluation of RNAi cotton plants grown in the USA environment in 2015-2016 also showed that the RNAi line had longer fiber than that of Coker 312 (Jenkins *et al.* 2017; unpublished results). The fiber length is generated through primary wall synthesis which dominates in 10-DPA fiber. Although the components of fiber cell wall change dynamically throughout the development, the primary walls of 10-DPA cotton fibers, similar to other expanding plant cells, mainly contain cellulose, pectin, xyloglucan, lignin and hydroxyproline-rich glycoprotein [32]. Extensin, one of the glycoproteins, is involved in the generation of scaffolding network of the cell wall [33]. In this study, a pollen ole e 1 allergen and extensin protein encoded by the gene Gh_D05G0805, was highly enriched in 10-DPA RNAi cotton

(Table 2 and Figure 5). The ole e 1 containing proteins were first identified as major allergens and present commonly in many plants, including cotton [34]. It was expressed not only in pollen but also in other plant tissues [35] [36] [37]. An ole e 1 containing protein encoded by AtAGP30 in Arabidopsis was shown to be required for root regeneration and seed germination [37]. It is reasonable to propose that extensin plays an essential role in primary cell wall architecture of cotton fiber just as in other plant cells.

It is tempting to speculate on the relationship of molecular changes associated with the cotton *PHYA1* RNAi phenotypes based on the results of altered molecular processes in the fiber of RNAi plants. Shifting from vegetative growth to reproductive growth, establishment of the seedling, and switching of the circadian clock are regulated by phytochrome [38] [39]. It was reported that *PHYA* plays an important role in seed germination under a broad spectrum of light and promoting flower under long day light condition in Arabidopsis [39]. A recent study showed that transgenic cotton plants (*G. hirsutum*) overexpressed the phytochrome B gene from *Arabidopsis thaliana* had increased plant growth and yield [38]. A previous report on biochemically purified Arabidopsis *PHYA* holoproteins had showed that the phytochrome molecule consisted of two structural domains connected via a flexible hinge region [39]. Recent studies on molecular mapping of fiber QTLs using the *PHYA1* CAPS (cleaved amplified polymorphic sequences) marker specific to hinge region of Phytochrome A1 with flanking SSR markers reported that the *PHYA1* CAPS marker was significantly linked to fiber length [15] [40]. It was found that one of the flanking markers linked to *PHYA1* CAPS was associated with micronaire and lint yield in cotton [41]. These results on the indirect association of the *PHYA1* CAPS marker with important fiber traits complements the results of differentially expressed genes in fiber and provide valuable information on the potential molecular basis of the cotton *PHYA1* RNAi phenotypes and fiber development.

Sucrose synthase (*Sus*) is the major enzyme of sucrose breakdown for cellulose biosynthesis in cotton (*G. hirsutum*) fiber [42]. Two genes Gh_A08G1031 and Gh_D08G1309 encoding sucrose synthase 1 (*Sus 1*) were down-regulated in RNAi line compared to Coker 312. It was reported that the expression level of *Sus 1* was low in 10 DPA fiber [43], which was perfectly consistent with the finding in this study. Although GhSus3 (U73588) has been demonstrated to play an important role in ovule development and fiber cell initiation, the functional characterization of *Sus* genes however was limited [43]. The development of fiber is a complex process, and there are some overlapping processes among each developmental stage. The transition from primary to secondary cell wall synthesis might occur in 10-DPA fibers. Sucrose synthase was reported to participate in secondary cell wall synthesis [44]. Therefore, the RNAi cotton line might have lagged in primary stage much longer than Coker 312, resulting in generating longer fiber. A study on the expression of *Sus 1* at all developmental stages (−3 DPA, 0 DPA, 5 DPA, 15 DPA and 20 DPA) would provide a better understanding of the function of *Sus 1* in fiber development.

In cotton, brassinosteroid (BR) positively affects fiber development [11]. It was found in this study that the gene Gh_D13G0889 encoding a cytosolic sulfo-transferase 12-like protein was up-regulated, and the gene Gh_D04G1245 for a bahd acyltransferase was down-regulated in the RNAi line. The Arabidopsis brassinosteroid sulfotransferase, a type of cytosolic sulfotransferase, was found more abundant in young seedlings and actively growing cell cultures, and its expression was induced in response to salicylic acid and methyl jasmonate and bacterial pathogens [45]. The dwarfism caused by overexpression of bahd acyltransferase can be rescued by brassinosteroid in Arabidopsis [46]. In addition, acylation conjugation of brassinosteroid catalyzed by bahd acyltransferase plays a role in brassinosteroid homeostasis. Together, it suggested that fiber development might be regulated by brassinosteroid metabolism and balancing in the *PHYA1* RNAi cotton.

Besides brassinosteroid, the phytohormone ABA is known to inhibit fiber initiation [10]. Gh_D08G1639 and Gh_A08G1344 both encoded an abscisic acid 8'-hydroxylase 1-like protein. The ABA 8'-hydroxylase catalyzed the first step of inactivation of ABA. Therefore, the active level of ABA might be increased by decreasing the expression of ABA 8'-hydroxylase in the RNAi line. These results are consistent with the physiology data that ABA concentrations increased from the day of anthesis to 10 DPA and then declined until 20 DPA [47]. The fiber development is a dynamic process; at the stage of 10 DPA, ABA inhibits fiber cell initiation and fiber cells are predominantly elongated in RNAi cotton.

It is well known that ethylene plays a major role in cotton fiber cell elongation [48]. In this study, six genes (Gh_D05G0148, Gh_D08G2484, Gh_A08G2112, Gh_A05G0434, Gh_D05G0562 and Gh_06G1046) encoding an EIN3-binding F-box protein 1(EBF1)-like protein, were down-regulated in RNAi cotton. The reduction of EBF1 activates ethylene response by releasing EIN3 and EIL1 proteins from ubiquitination and subsequent proteasomal degradation [49]. In addition, six genes (Gh_D05G3348, Gh_D10G0255, Gh_D06G1403, Gh_D01G0450, Gh_A04G0305 and Gh_A06G1144) encoded ethylene-responsive transcription factor 4 (ERF4)-like proteins were down-regulated in the RNAi line. In Arabidopsis, AtERF4 is a negative regulator, capable of modulating ethylene and abscisic acid responses [50]. The reduction of ERF4-like proteins might promote fiber development through activating ethylene signaling pathway mediated by EIN3. Thus, ethylene signaling pathways might also contribute to fiber elongation in RNAi cotton as well as its early maturity characteristics. Additionally, many genes encoding WRKY or probable WRKY transcription factors were down-regulated in *PHYA1* RNAi cotton, which was consistent with the observation of Wan *et al.* (2014) [51], in that WRKY family transcription factors were highly expressed in naked seed or fuzzless cotton mutants [51].

4.2. The Possible Functions of Cytochrome P450 in Cotton Fiber Improvement

Plant cytochrome P450s have been shown to play an important role in response

to environmental stimuli and participate in the synthesis of a variety of biomolecules including fatty acid conjugates, plant hormones, secondary metabolites, lignin, and defensive compounds [52]. It was reported that *PHYA1* RNAi plant had a better tolerance to abiotic stresses, including salinity, heat, and drought stresses with 10% - 18% yield improvement [7].

The results of KEGG analysis implied that cytochrome P450 involved pathways might play a role in fiber development in the RNAi line and explain yield improvement due to stress tolerance. In addition, a miRNAome analysis for the *PHYA1* RNAi line showed that cytochrome P450 TBP (TATA Binding Proteins) have the highest hits in miRNA target prediction [53]. Cytochrome P450s were involved in almost every aspects of the plant life [54]. Based on evolutionary analyses, the oldest CYPs are involved in biosynthesis of carotenoid and sterol. The middle branches of CYPs mediate the adaptation to environment, including abiotic stresses and biotic defenses. The newest CYPs are involved in the synthesis of structural components such as lignin, fatty acid, pigments, odorants and signaling compounds [54]. Besides with improved fiber quality, *PHYA1* RNAi plants have vigorous root and vegetative growth, and early flowering due to increased photosynthesis [6] [7]. CYPs are light-driven oxidase enzymes in electron transfer chains [55]. This study thus supports the hypothesis that increased photosynthesis of RNAi cotton might be via miRNA-mediated expression regulation on CYPs. In addition, CYPs are also involved in biosynthesis and catabolism of many plant hormones, such as ABA (CYP707), GA (CYP714) and BR (CYP724) [54]. ABA regulates osmotic stress tolerance [56], and GA plays a role in salinity adaptation [57]. As mentioned previously, CYPs also play crucial roles in environmental adaptation, which might explain the better tolerance of RNAi cotton to salinity, drought and heat stresses as well as increase of potential and operative yields.

5. Conclusion

RNA-seq based comparative transcriptome profiles of 10 DPA fiber tissues of *PHYA1* RNAi lines and its wild-type genotypes not only revealed in-depth insights into the key molecular signatures and altered genetic pathways, explaining improved fiber characteristics in the genetic background of cotton *PHYA1* gene interference, but also indirectly explained a better environmental adaptation, improved flowering and maturity and yield potential of RNAi lines. Results should be helpful in future fine-tuning and re-programming of fiber development through targeted engineering of characterized signatures of key genetic pathways or their combinations using new generation of genome modification and editing tools.

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