Communication

Proteomic profiles of cotton fiber developmental transition from cell elongation to secondary wall deposition

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Abstract: Cotton fiber development transition from elongation to secondary cell wall biosynthesis is a critical growth shifting phase that affects cotton fiber final length, strength and other properties. Morphological dynamic analysis indicates that an asynchronous fiber developmental pattern between two cotton species. The critical time point for *Gh* and *Gb* fiber elongation termination is, respectively, 23 and 27 days post-anthesis (dpa). The temporal changes of protein expression at three representative development periods (15-19, 19-23, 23-27 dpa) were examined in both species with iTRAQ technics. Strikingly, a large proportion of differentially expressed proteins (DEPs) was identified at 19-23 dpa in *Gh* or at 23-27 dpa in *Gb*, corresponding to their fiber developmental transition timing from elongation to secondary cell wall biosynthesis. To better understand fibers transitional development, we comparatively analyzed those DEPs in 19-23 dpa of *Gh* vs. in 23-27 dpa of *Gb,* and noted that these cotton species indeed share fundamentally similar fiber development features under the biological processes. It also showed that there have limited overlaps in both specific upregulated and downregulated proteins between the two species, suggesting specie-specific protein regulations in development. Proteomic profiling revealed dynamic changes of several key proteins and biological processes that potentially correlate with fiber development transition. During the transition, upregulated proteins mainly involved in carbohydrate/energy metabolism, oxidation-reduction, cytoskeleton, protein turnover, Ca^{2+} signaling etc, whereas important downregulated proteins mostly concentrated in phenylpropanoid and flavonoid secondary metabolism pathways. Several changed proteins in this key stage were also validated by qRT-PCR. Overall, the present study provides accurate pictures of the regulatory networks of functional proteins during the fiber developmental transition.

Keywords: developmental transition; proteomics; *Gossypium hirsutum*; *Gossypium arbadense*

1. Introduction

Cotton (*Gossypium* spp.) is one of the most important crops, contributing prevalent natural textile fiber worldwide. The most commonly cultivated cotton species today are upland cotton (*G. hirsutum.*, *Gh*) and sea island cotton (*G. barbadense*, *Gb*), both of which are tetraploid plants originated from interspecific hybridization event about 1-2 million years ago [1]. Selective breeding of the cottons has led to the high yield and diverse environmental adaptability of *Gh*, therefore the *Gh* cultivation now

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accounts for the majority of cotton fiber production, whereas the *Gb* grown in selected environments is prized for superior fiber length, strength, and fineness [1]. Nowadays, within the premium textiles market, there is a demand for higher-quality, high-yield cotton fibers, however the molecular mechanics governing fiber quality are still not well understood. Unraveling the molecular basis for different fiber agronomic traits between Gh and Gb will contribute significantly to the characterization and manipulation of the specific genes that control fiber quality and yield, thereby allowing for the improvement of cotton fibers.

Cotton fibers are highly elongated and thickened seed epidermis single cells that undergo four major sequential and overlapping developmental stages: fiber initiation, elongation (primary cell wall synthesis), cell wall thickening (secondary cell wall deposition) and maturation [2]. Given that the cotton fiber quality is determined by the final length and strength, many endeavors have devoted to investigating the regulatory mechanisms underlying the fiber cell elongation and secondary cell wall deposition during cotton development. In recent years, knowledge of cotton genome structures, interrelationships between cotton varieties in relation to development and evolution have obtained impressive increases by using multi-omics analysis approaches [3].

Proteins are the direct performers for most biological activities and functions. Proteomic analysis that provides overall information about protein regulation and active pathways has been widely used to reveal molecular mechanisms of particular biological processes [4]. Amongst the various proteomic technologies, the "isobaric tags for relative and absolute quantitation" (iTRAQ) method can identify numerous proteins and provide more reliable quantitative information than conventional analysis by two-dimensional gel electrophoresis [4]. Prior studies using iTRAQ analysis have gained insight into the differences in gene/protein expression of cotton in response to environment stress [5], and domestication [6], as well as fiber development [7-9].

The fiber developmental transition stage is often thought of as a period when primary and secondary wall deposition overlap [10]. During the transition, fiber development is accompanied with significant changes in physiological processes and cell wall protein contents, requiring organization and rearrangements of polysaccharides. Here, we have identified a significant variation at the timing of development transition among different cotton species by fiber morphological and proteomic dynamic analysis. Based on comparative proteomic analysis, we revealed two cotton species, *Gh* and *Gb*, share a highly similar development regulatory patterns during their respective fiber cell transition. Furthermore, several key interspecific differentially regulated proteins that are potentially involved in the cotton fiber developmental transition stage were identified and analyzed. This study provides new clues concerning the fiber development transition at proteomic level, thereby highlighting candidate genes/proteins and related pathways for cotton fiber improvement.

2. Experimental procedures

2.1. Measurement of fiber length and thickness

Two cotton cultivars, Gh cv. Xinluzao 36 and Gb cv. Xinhai 2, were healthily grown in fields at the Xinjiang Horticulture Experimental Station. Flowers were tagged at anthesis, and developing bolls were harvested every 4 days in an interval from 11 to 35 dpa. The bolls were dissected immediately, and the length of fibers were determined using a previously reported method [11]. To determine the fiber thickness, fibers were fixed in 3% glutaraldehyde and dehydrated in an ethanol series (from 30% to 100%) before then being infiltrated with Spurrs resin (Electron Microscopy Sciences). The thickness of the cell wall was examined by measuring the cross-section fibers under a transmission electron microscope (TEM) (Bio-TEM H-600, Hitachi, Japan). All measurements were conducted with at least 100 fibers and 10 different ovules at corresponding boll age.

2.2. Protein extraction of cotton fiber

Cotton fiber protein extractions were performed using a modified phenol extraction method as reported [12]. For protein extraction, a total of 24 independent *Gh* and *Gb* samples were collected at time points 15, 19, 25, and 27 dpa. Approximately 800 mg fibers were finely ground with liquid

nitrogen, and then was homogenized in 5 ml buffer (50 mM Tris-HCl, pH 8.0, 30% sucrose, 2% SDS, 1% DTT, and 1mM PMSF). After adding saturated phenol, the mixture was vortexed and centrifuged at 10,000 ×g for 10 min at 4 ℃. The upper phenol phase was collected and mixed with 5 ml NH_4AC (0.1 M) at -20 ℃ for 30 min, and then centrifuged at 20,000 ×g for 20 min at 4 ℃. The pellets were collected and washed with cold NH_4AC (0.1 M), and 80% acetone to obtain proteins. The proteins were vacuum dried and stored at -80℃.

2.3. Proteome analysis of cotton fibers by iTRAQ

Fiber proteomic data were collected at the National Center for Protein Sciences Beijing, China. Before labeling, the total protein of each sample (100 μg) was digested and reconstituted using 8-plex iTRAQ reagent (AB Sciex Inc., CA, USA). The fiber proteins of *Gh* at the developmental time point of 15, 19, 23 and 27 dpa were labeled with iTRAQ tags 114, 116, 118 and 121, and those of *Gb* were labeled with iTRAQ tags 113, 115, 117, and 119, respectively. Chromatography consisted of Thermo Surveyor HPLC system was operated at 500 nL per minute via a split solvent line. Each sample was loaded on a BioBasic C18 reversed phase column (Thermo 72,105-100,266) and flushed for 20 min with 5% acetonitrile (ACN), 0.1% formic acid to remove salts. Peptide separation was achieved using a Thermo Surveyor MS pump with a gradient HPLC method washing from 5% ACN to 50% ACN in 620 min, followed by a 20 min wash of 95% ACN and equilibration with 5% ACN for 15 min. The Surveyor was coupled with a Thermo LCQ DECA XP Plus mass spectrometer with a stock nanospray ion source. Data were acquired with a 2.5 kV ion spray voltage, 30 PSI curtain gas, 5 PSI nebulizer gas, and 150 ℃ interface heater temperature. Each cycle time was fixed to 2.5 s. Dynamic mass exclusion windows were 2 min long with a repeat count. All samples were run with three replicates.

2.4. Protein identification and bioinformatical analysis

Protein identification and quantification were performed using the Mascot 2.3.02 software (Matrix Science, Boston, USA). MS/MS spectra were analyzed with Protein Pilot software (Protein Pilot 4.0; AB SCIEX) against the corresponding genome databases using the Paragon algorithm. An automatic decoy database search strategy was employed to estimate the false discovery rate (FDR) using the PSPEP software integrated in the Protein Pilot. Proteins were identified using the following parameters: sample type = iTRAQ 8-plex (peptide-labeled), Cys; alkylation = iodoacetamide; digestion = trypsin; instrument = Triple TOF5600 (AB SCIEX). To annotate coding sequences with the highest score, we searched the non-redundant protein sequence database at NCBI. Differential expression from the protein data was judged with the following criteria: number of unique peptides≥2; threshold of fold change for upregulation/downregulation = 1.5/0.67; and maximum allowed fold change = 30. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with identified differentially expressed proteins[13, 14]. Once $p < 0.05$, the GO term or pathway was regarded as a significant enrichment.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from fiber samples using a Total RNA Isolation Kit (Biorbyt, San Francisco, United States). First strand cDNAs were synthesized from 1 μg of total RNA using the ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). The reverse transcription product was diluted 30-fold with RNase-free water and stored at -80 °C. The specific primers for selected genes were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Cotton Ubiquitin7 gene was used as a reference gene to normalize the cDNA amplification in each reaction. Triplicate replicates of qRT-PCR were performed with SYBR Premix Ex Taq (TaKaRa, Dalin, China) on ABI 7500-Fast Real Time PCR system (Applied Biosystems, CA, USA). Relative gene expression levels were calculated using the $2^{-\Delta CT}$ method. The amplification primers are listed in Supplemental Table 2

3. Results and Discussion

3.1. Morphological dynamic analysis of cotton fiber development

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Cotton fiber cells undergo substantial elongation and expansion throughout development. To investigate the interspecific divergence of fiber development between *Gh* and *Gb*, we determined the fiber lengths across from 5 to 37 dpa. The fiber length approximately linearly increased over the 5-19 dpa in *Gh* or 5-23 dpa in *Gb*, respectively. The elongation of *Gh* fiber almost ceased after 23 dpa, while the elongation in *Gb* fibers ceased around 27 dpa (**Fig. 1A**). The elongation rate (length increase per day) within each species declined sharply after 19 dpa, whereas a higher fiber elongation rate occurred in Gb at later periods (**Figure 1B**). This identified prolonged fiber elongation in *Gb* cotton, consistent with prior reports [10]. Plant cell wall thickening usually is the effect of secondary cell wall biosynthesis. Fiber cell wall thickness was also examined using TEM. It showed that the fiber cell wall thickness has no apparent difference between two cotton species during the fibers rapid elongation period. Fiber cell wall thickening initiation in *Gh* cotton was at 19 dpa, beyond that time point the cell wall thickness dramatically increased, but the onset of cell wall thickening in *Gb* fibers had a delay, starting at around 23 dpa (**Figure 1C**). Compared with *Gh*, a faster thickening rate was also found in *Gb* during subsequent fiber growth (**Fig. 1D**).

These results suggested that two respective periods, 19- 23 dpa and 23-27 dpa, were crucial for Gh and Gb development respectively, involving the transition phase from elongation to secondary cell wall deposition. Prior studies of the two near-isogenic cotton lines showed that the transition to secondary wall deposition correlates with their fiber bundle strength differences, and the duration of this transition stage may determine cotton fiber length, as well as other properties [15]. Importantly, the prominent phenotypic differences between these two cotton species does suggest an asynchronous fiber developmental pattern in transition from elongation to secondary cell wall deposition.

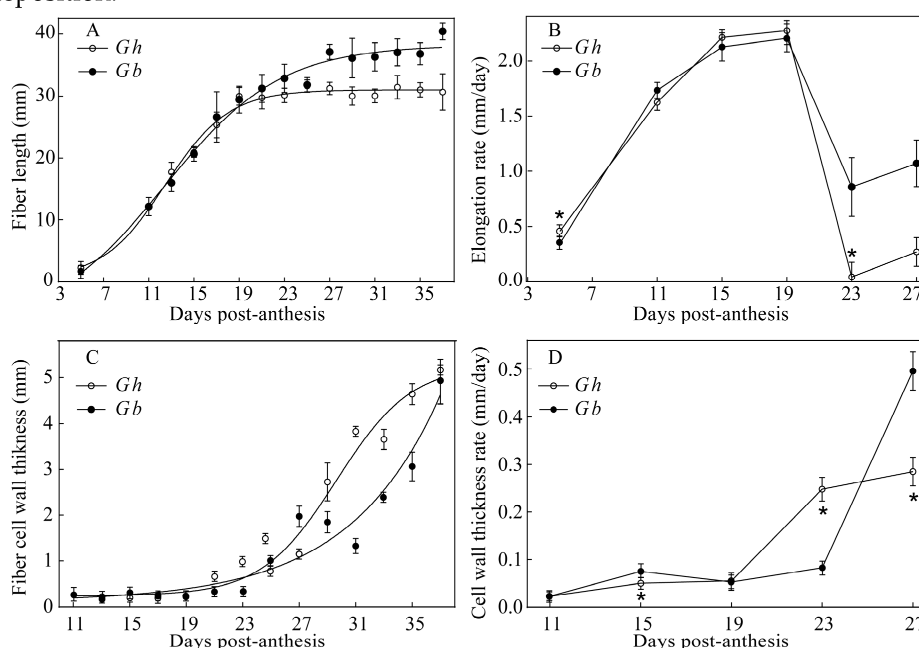

Figure 1. The dynamic change of fiber length and cell wall thickness during development. (A). *Gh* and *Gb* cotton fiber length at different dpa. Standard deviation (SD) of error bars were calculated with fifteen biological replicates. (B). *Gh* and *Gb* cotton fiber elongation rate across development time. (C). *Gh* and *Gb* cotton fiber cell wall thickness at different dpa. Approximately 30 fibers were measured for each sample. (D). *Gh* and *Gb* cotton fiber cell wall thickness rate across time.

3.2. Proteomic analysis of cotton fiber development

To shed light on the mechanisms controlling fiber development, we examined proteome changes during fiber rapid elongation and structurally thickening stages. The eight-plex iTRAQ experiments allowed for a detailed comparison of cotton fiber protein expression differences at adjacent periods (15-19, 19-23, and 23-27 dpa). A total of 1197 proteins were successfully identified at a 95% confidence level and a 1.0% FDR. Finally, we quantified and annotated 797 distinct proteins with two or more

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unique peptides by using cotton databases [16, 17]. Proteins that showed a difference in abundance corresponding to at least a 1.5-fold change and a P value of < 0.05 were considered to be differentially expressed proteins (DEPs). Based on these criteria, 112 and 94 DEPs were identified in *Gh* and *Gb*, respectively. A total of 102 unique DEPs (12.7 % of 797 proteins) among two cotton species were identified at those adjacent developing time points

To classify these DEPs, we performed the GO term enrichment analysis, which was divided into molecular function, biological processes, and cell composition (**Fig. 2** and **Supporting Information Table S1**). Biological terms having significantly enriched (false discovery rate, FDR-corrected < 0.01) were shown. In molecular function class, ion binding (36.0% of the total number of DEPs) and small molecule binding (23.1%) and oxidoreductase activity (22.4%) are the three most abundant subcategories, followed by hydrolase activity (17.5%), carbohydrate derivative binding (16.6%), structural constituent of ribosome (12.0%), cofactor binding (8.8%), lysase activity (6.2%) and isomerase activity (3.9%). Similarly enriched molecular function classes of DEPs have been reported in previous studies on cotton fiber development [9]. In biological process category, organic substance metabolic process (59.7%), primary metabolic process (56.8%), cellular metabolic process (55.5%), single-organism metabolic process (41.6%) presented the most significant four enrichment among others. Given that the enriched proteins mainly involved in various metabolic processes, the regulation of the basal metabolic reactions is believed to play a critical role in sustaining a rapid developmental growth of cotton fiber cell. In the cellular component category, cell part (52.3%) was the most abundant subcategory, followed by intracellular (50.7%), membrane-bounded organelle (23.7%) et al (**Fig 2.**), indicating cotton fiber development mainly involved regulation of intracellular proteins at these stages.

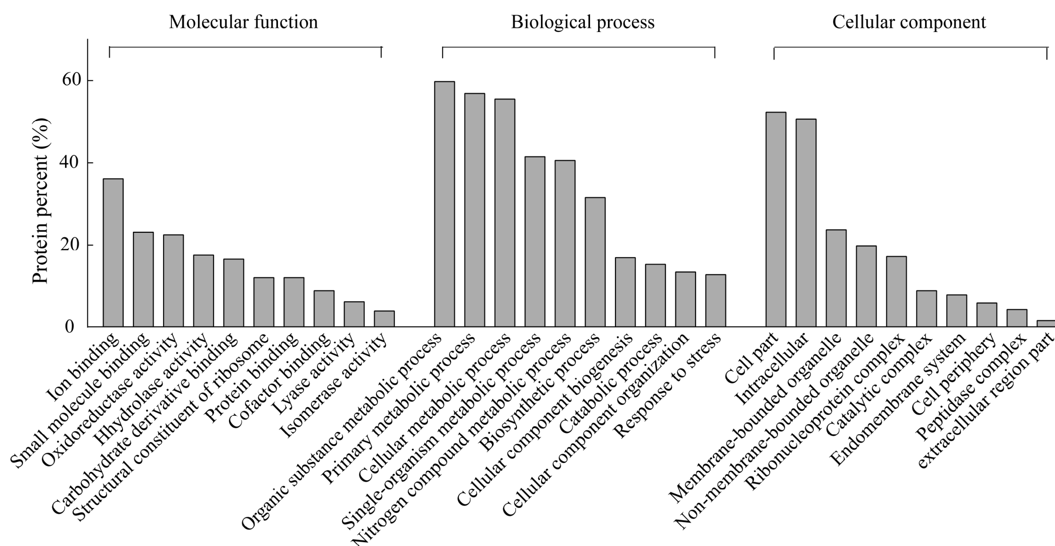

Figure 2. Gene Ontology analyses of differentially expressed proteins during fiber cell development. The ten most predominant enriched terms are shown in the GO categories.

To comprehend pathways in the fiber development, we also preformed KEGG enrichment analysis on those DEPs. It showed that several key metabolism and protein-related biochemical pathways were significantly enriched (FDR < 0.01) **(Fig. 3**). The enrichments are mainly associated with metabolic pathways (30.8%), biosynthesis of secondary metabolites (22.1%), carbon metabolism (13.6%), ribosome (12.0%), glycolysis/gluconeogenesis (8.4%), citrate cycle (TCA cycle) (6.2%), glutathione metabolism (6.2%), biosynthesis of amino acids (6.2%), pyruvate metabolism (5.8%), protein processing in endoplasmic reticulum (5.2%), proteasome (4.2%) and peroxisome (3.6%) (Fig 3.). The distribution of the differentially regulated proteins indicates a strong role of energy/carbohydrate metabolism pathway throughout fiber development, as well as remarkable secondary metabolic pathway regulations involved. This result is plausible, because rapid cell elongation and fiber development require a large amount of energy and carbon intermediates for cell wall synthesis [18].

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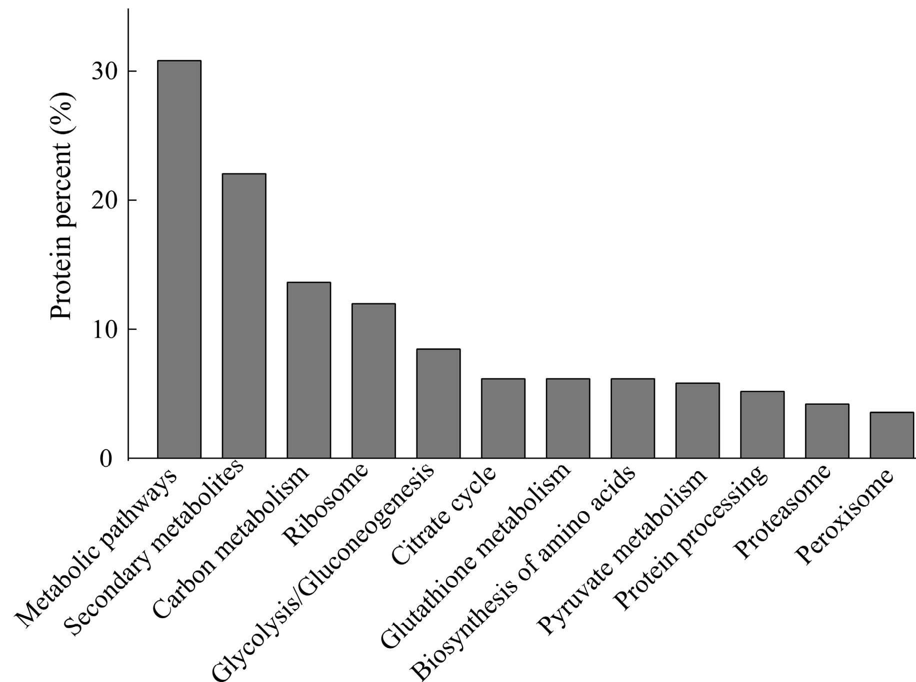

Figure 3. KEGG pathway enrichment analysis of differentially expressed proteins during the fiber cell development. The twelve most significantly enriched pathways were shown.

3.3. Identification of the cotton fiber developmental transition stages at protein-level

Upon further inspection of the identified DEPs across 15-19, 19-23 and 23-27 dpa for both species, we noted that the DEPs quantities are unequally distributed between the adjacent time points in each cotton specie (**Fig. 4**). Twenty (15-19 dpa), 57 (19-23 dpa), and 35 (23-27 dpa) DEPs were identified in *Gh*, and 23 (15-19 dpa), 23 (19-23 dpa) and 48 (23-27 dpa) DEPs were found in *Gb*, respectively. There is a distinct developmental period in both cotton species where the identified DEPs were almost double in comparison with those of all other periods, representing a burst stage in protein differential regulation (**Fig 4.**). Interestingly, the identified largest amount of DEPs was between 19 and 23 dpa in *Gh*, having a total of 57 DEPs, whereas the maximum number of DEPs was instead of presenting at the same time intervals, and that happened between 23 and 27 dpa in *Gb*, with 48 DEPs (**Fig 4.**) Strikingly, the delayed burst in DEPs in *Gb* is consistent with the morphological dynamic analysis showing that *Gb* fiber development exhibited a delayed onset in secondary wall deposition. Moreover, it has been reported that there have a large number of cotton gene regulations at transcription level in fiber developmental transition, usually in 19-27 dpa [19]. During this distinct transition stage, cotton fiber development experiences some important physiological and biochemical events occurring, for example, changes of metabolic sugar contents [20], the degradation of the cotton fiber middle lamella [21], the deposition of winding cell wall layer [22]. Therefore, it is presumed that there must be a marked change in protein expression pattern associated with the transition stage of fiber development [19]. Identification of the significant proteomic variations within cotton fibers switching to secondary cell wall deposition indicates that the critical transition stage can be distinguished by protein expression dynamics. Based on these analyses, *Gh* and *Gb* cotton undergo the asynchronous fiber developmental transition process at protein level. Cotton fiber development transition is a significant shifting phase and is worthy of further investigation to discover critical developmental factors responsible for fiber length, stiffness and strength [23].

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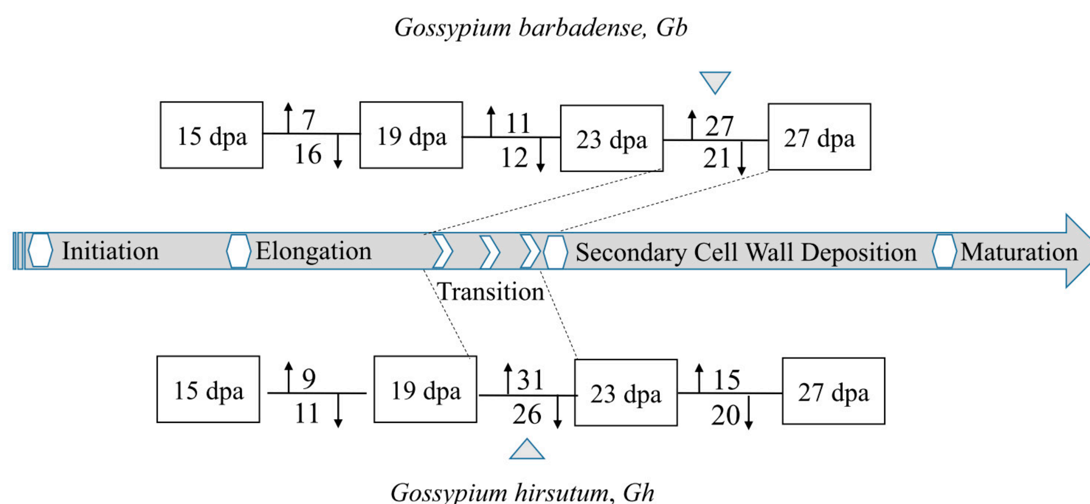

Figure 4. Temporal changes of protein expression among two cotton species developing fibers. Numbers that beside of the arrow line designate the number of up-regulated/down-regulated proteins (at least 1.5-fold and FDR < 0.05) relative to their adjacent developmental time points. The highest number of differentially expressed proteins occurred in the transition to secondary cell wall synthesis.

3.4. Comparative analysis of differentially expressed proteins during fiber developmental transition between two cotton species

The fiber development transition experiences extreme morphological changes from elongation to cell wall thickening and generates significant protein expression variation among *Gh* and *Gb*. To gain insight into the cotton fiber development transition, the DEPs from these distinct periods for both species (19-23 dpa for *Gh* vs. 23-27 dpa for *Gb*) were profiled and compared on basis of biological process. In the stage, 31 upregulated and 26 downregulated proteins were identified in *Gh* cotton, while 27 upregulated and 21 downregulated members were recorded for Gh cotton fibers. Notably, we found that *Gb* and *Gh* indeed shared a highly similar development when these two stages, 19-23 dpa for Gh and 23-27 dpa for Gb, were compared under the biological processes. Function comparative analysis revealed changes in several key biological processes, identified by DEP number in each category, with significant similarity between the two species. In both cottons, the upregulated proteins were mainly involved in carbohydrate metabolism, oxidation-reduction, cytoskeleton organization, response to calcium ion, proteolysis, glycolysis, lipid metabolism, signal transfection, protein folding and transport process, whereas the downregulated proteins were mainly involved in ribosome biogenesis, secondary metabolism, signal transduction, nucleic acids processing, protein folding and amino acid metabolic processes, among others (**Fig. 5**).

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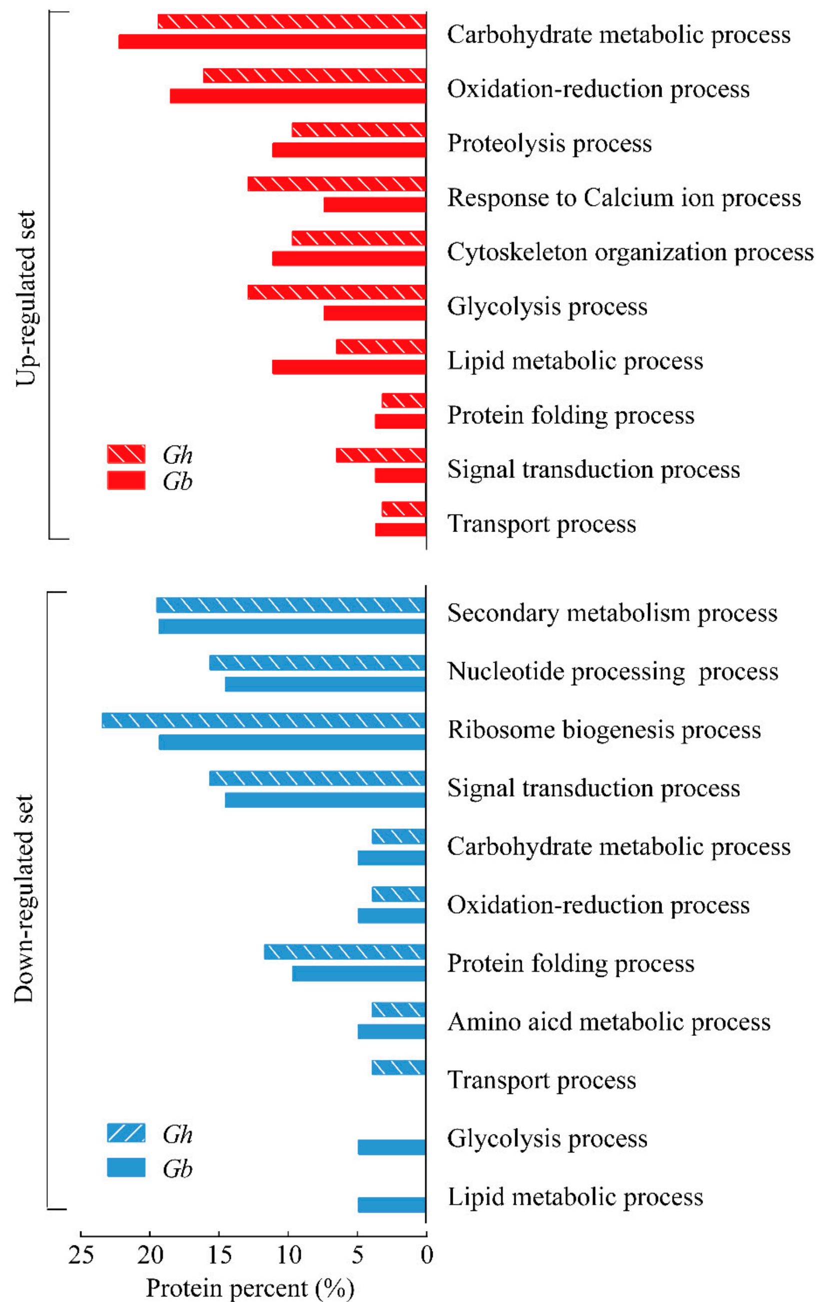

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262 **Figure 5.** Comparative analysis of differently expressed proteins under biological processes among respective
263 protein regulation burst periods of two cotton species. The identified differently expressed proteins were
264 classified into up-regulated and downregulated protein sets, and then carried out the GO biological processes
265 annotation.

266 Furthermore, a pairwise comparison with DEPs that identified in the two cotton species
267 respective transitions was undertaken to explore protein expression patterns. All identified DEPs
268 from this key development stage were summarized and listed in Tables 1. Eighteen common up-
269 regulated and 15 common down-regulated proteins were found in two cotton species (**Fig. 6**). These
270 common proteins account for 58.1%, 66.7% of upregulated proteins and 57.7%, 71.4% of
271 downregulated proteins in *Gh* and *Gb*, respectively. This suggests that two cotton species have only
272 partial overlap in their fiber-developmental proteome during the transition, with some protein
273 regulation being unique to both species during fiber development. This expressed protein variation
274 could be responsible for interspecific phenotypic differences that include, for example, fiber traits.

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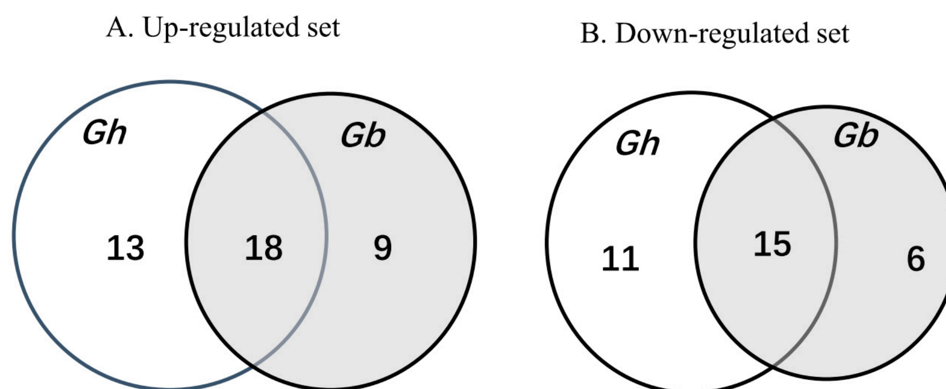

Figure 6. Venn diagram analysis of the differentially expressed proteins between *Gh* and *Gb* cotton during fiber development transition from elongation to second cell wall deposition. Venn diagrams showing the number of differentially expressed proteins and the overlap of identified common regulated proteins among *Gh* and *Gb* cotton during the fiber cell wall developmental transition.

3.5. Key gene/proteins and pathways involved in the cotton fiber developmental transition

During the developmental transition between primary and secondary wall deposition, the cotton fibers undergo spatial and temporal cell wall remodeling. In our identified protein categories, the largest number of upregulated proteins were involved in carbohydrate metabolisms. Carbohydrate metabolism pathway provides the essential carbon skeletons for the synthesis of cell wall polysaccharides and fatty acids, as well as energy storages [18]. Several remarkably upregulated common proteins in carbohydrate metabolism pathways have been identified in both *Gh* and *Gb* cotton species, including sucrose synthase (SUS), sucrose synthase-like (SUSL), cellulose synthase 8 (CESA8), endoglucanase (EG), and pectinesterase (PME), which have all been reported to be involved in cell wall biogenesis and important for cotton fiber production and quality [24, 25]. It is noting worth that of those DEPs, the PME is one of the most substantially regulated proteins, having a 11.29 and 17.45-folds increase in *Gh* and *Gb*, respectively (Table 1). Indeed, plant PME catalyzes the de-esterification of pectin, one major component of the primary cell wall and middle lamella, has been shown to play crucial roles in regulating cell wall expansion, elongation and adhesion [25]. Compared with *Gh*, a higher-fold PME upregulation occurred in *Gb* fibers at the transition phase, consistent with prior reports that a higher PME activity was found during the later stages of cotton fiber development [25]. In *Gb* cotton fibers, other several key carbohydrate metabolism pathway enzymes, SUS, SUSL, EG and CESA8 also showed much more folds expression upregulation, suggesting *Gb* fiber transition has a superior carbohydrate metabolism than the transition in *Gh*. Meanwhile, several proteins distinct to both species were detected in proteomic analysis, such as upregulated UDP-D-glucose dehydrogenase in *Gb*, and reinforced expressed acid beta-fructofuranosidase-like protein in *Gh*. These results suggest that stage-specific, upregulated critical enzymes in carbohydrate metabolism pathways would facilitate fiber cell wall developmental transition and might contribute to the variation in fiber traits between species. Furthermore, secondary wall cellulose micro-fibril formation in cotton fiber cells is an energetically costly process [2]. Multiple proteins involved in glycolysis, TCA cycle, including enolase, ADP/ATP carrier proteins, pyruvate dehydrogenase E1 component subunit beta-3 and glyceraldehyde-3-phosphate dehydrogenase, presented in the up-regulation category, suggesting that 'energy' production is still active cellular process at fiber transition stage and might be a basis of the fiber physiological changes.

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309 310 **Table 1.** Functional categorization and comparative analysis of changed proteins involve in fiber important developmental transition among two cotton species.

Acc.ID	Protein name	Fold change		Putative functions
		Gh (19-23dpa)	*Gb* (23-27dpa)	
Common up-regulated proteins				
A0A1U8JM46	Sucrose synthase-like(SUSL)	1.52	1.93	Carbohydrate metabolism
G1FNX7	Sucrose synthase (SUS)	1.80	3.37	Carbohydrate metabolism
A0A1U8J0C9	Endoglucanase(EG)	2.03	3.56	Carbohydrate metabolism
P93155	Cellulose synthase 8 (CESA8)	3.43	5.45	Carbohydrate metabolism
A0A1U8JXD0	Pectinesterase (PME)	11.29	17.45	Carbohydrate metabolism
A0A1U8MZ87	L-ascorbate peroxidase(APX)	1.55	1. 71	Oxidation-reduction
I7CUD1	NADP-dependent D-sorbitol-6-phosphate dehydrogenase-like (S6PDH)	1.80	1. 63	Oxidation-reduction
A0A1U8L9Z6	L-ascorbate peroxidase 6(APX6)	2.25	1.79	Oxidation-reduction
A0A1U8N003	Protein aspartic protease 1-like	1.60	1.51	Proteolysis
A0A1U8LFS6	Proteasome subunit beta type(PSB)	4.62	2.05	Proteolysis
A0A1U8I8H1	Carboxypeptidase Y-like(CPY)	2.75	2.31	Proteolysis
A0A1U8J5B7	Calmodulin-7(CaM7)	1.55	1.63	Response to calcium ion
A0A1U8HKZ3	Calreticulin-like(CRTL)	1.55	1.81	Response to calcium ion
D2D2Z9	Annexin（ANN）	2.54	3.81	Cytoskeleton organization
Q6VAF8	β-tubulin-3（TUB-3）	2.76	1.81	Cytoskeleton organization
A0A1U8N0A9	Enolase(E)	1.86	1.82	Glycolysis
A0A1U8ND61	ADP, ATP carrier protein 3	2.28	1.76	Glycolysis
A0A1U8K7G6	Phospholipase D (PLD)	1.76	2.95	Lipid metabolism
Common down-regulated proteins				
A0A1U8IHV5	Shikimate-hydroxycinnamoyltransferase-like (HCTL)	0.61	0.56	Secondary metabolism
A0A1U8NC64	Naringenin(NAR)	0.43	0.52	Secondary metabolism
A0A1U8JD61	Anthocyanidin reductase-like(ANRL)	0.61	0.47	Secondary metabolism
D6N3G6	Chalcone-flavanone isomerase family protein(CHI)	0.51	0.40	Secondary metabolism
A0A1U8LZK4	Histone H2A	0.58	0.61	Nucleic acids processing
A0A1U8M305	Histone H4	0.41	0.56	Nucleic acids processing
A0A1U8NTG3	RNA polymerase II degradation factor 1-like	0.61	0.64	Nucleic acids processing
A0A1U8N216	60S ribosomal protein L8-3-like(RPL8-3L)	0.35	0.65	Ribosome biogenesis
A0A1U8NNS8	40S ribosomal protein S14-3-like(RPS14-3L)	0.59	0.58	Ribosome biogenesis
A0A1U8P6Y6	60S ribosomal protein L18a(RPL18a)	0.57	0.56	Ribosome biogenesis
A0A1U8I6P0	ERBB-3 Binding protein 1	0.61	0.57	Signal transduction
F8TRV3	14-3-3-like 2 protein	0.63	0.62	Signal transduction
A0A1U8M7X9	Glucan endo-1,3-beta-glucosidase 8-like	0.42	0.66	Carbohydrate metabolism
Q8L5K5	Fiber cell elongation protein *Gh*fe1	0.30	0.62	Oxidation-reduction
A0A1U8I0J1	10 kDa chaperonin-like	0.55	0.65	Protein folding
Different up-regulated proteins				
A0A1U8IHV5	Calcium-dependent protein kinase1 (CPK1)	1.68	N.D	Response to calcium ion
A0A1U8N4C1	Calcium-binding protein CML13	1.69	N.D	Response to calcium ion
A0A1U8HZ45	Peroxygenase 3	1.79	N.D	Oxidation-reduction
A0A1U8HU38	Monodehydroascorbate reductase-like(DHAR)	1.85	N.D	Oxidation-reduction
A0A1U8M685	Acid beta-fructofuranosidase-like	1.66	N.D	Carbohydrate metabolism
A0A1U8KR46	Cyclase-associated proteins（CAPs）	2.02	N.D	Cytoskeleton organization
A0A1U8LZK4	Pyruvate dehydrogenase E1 component subunit beta-3(PDC-E1B3)	2.06	N.D	Glycolysis

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A0A1U8HIS5	Glyceraldehyde-3-phosphate dehydrogenase(GAPDH)	1.55	N.D	Glycolysis
A0A1U8HGN1	Very-long-chain 3-oxoacyl-CoA reductase 1-like	2.66	N.D	Lipid metabolism
A0A1U8JCI9	Hsp70-Hsp90 organizing protein 3-like	1.60	N.D	Protein folding
A0A1U8MZI6	Guanosine nucleotide diphosphate dissociation inhibitor(GDI)	1.82	N.D	Signal transduction
A0A1U8IIZ3	Sulfurtransferase	1.52	N.D	Signal transduction
A0A1U8LGE5	Mitochondrial dicarboxylate transporter	1.56	N.D	Transport
A0A1U8IQ93	Malonate--CoA ligase-like	N.D	1.75	Lipid metabolism
Q9M6B8	Non-specific lipid-transfer protein	N.D	1.63	Lipid metabolism
A0A1U8IMR7	Enoyl-[acyl-carrier-protein] reductase	N.D	1.56	Oxidation-reduction
A0A1U8IHV5	Benzoquinone reductase	N.D	1.65	Oxidation-reduction
D2D2Z3	UDP-D-glucose dehydrogenase (UDPGDH)	N.D	1.74	Carbohydrate metabolism
A0A1U8JHY4	Profilin	N.D	2.43	Cytoskeleton organization
A0A1U8JD61	Fasciclin-like arabinogalactan protein 1(FLAs1)	N.D	3.30	Signal transduction
D6N3G6	Vacuolar protein sorting-associated protein	N.D	1. 93	Transport
Q8LK52	20 kDa chaperonin	N.D	1.83	Protein folding
Different down-regulated proteins				
A0A1U8L3Q2	60S ribosomal protein L21-1(60SRPL21-1)	0.61	N.D	Ribosome biogenesis
A0A1U8JSD0	40S ribosomal protein S16(RPS16)	0.62	N.D	Ribosome biogenesis
A0A1U8LK44	60S ribosomal protein L22-2(RPL22-2)	0.44	N.D	Ribosome biogenesis
A0A1U8NZL1	Activator of 90 kDa heat shock protein homolog	0.39	N.D	Protein folding
A0A1U8HMA6	14-3-3 protein 6-like	0.63	N.D	Signal transduction
B1NHU5	Fasciclin-like arabinogalactan protein 1(FLAs1)	0.61	N.D	Signal transduction
A0A1U8MRN1	STS14 protein-like	0.53	N.D	Signal transduction
A0A1U8NSL2	Cinnamyl alcohol dehydrogenase 9(CAD9)	0.55	N.D	Secondary metabolism
A0A1U8JC30	Fumarylacetoacetase-like(FALL)	0.56	N.D	Amino acid metabolism
A0A1U8M838	Nucleoside diphosphate kinase(NDPK)	0.44	N.D	Nucleic acids processing
A0A1U8ID75	Vesicle-fusing ATPase-like	0.65	N.D	Transport
A0A1U8L9M3	Pyruvate kinase(PK)	N.D	0.51	Glycolysis
A0A1U8PQ91	60S acidic ribosomal protein P2-1-like	N.D	0.64	Ribosome biogenesis
A0A1U8NAM1	Glutamate decarboxylase(GAD)	N.D	0.61	Amino acid metabolism
A0A1U8LIQ2	Non-specific lipid-transfer protein	N.D	0.57	Lipid metabolism
A0A1U8N0D2	Ras-related protein RABA1f-like	N.D	0.52	Signal transduction
A0A1U8NV91	Luminal-binding protein 5-like	N.D	0.51	Protein folding

311 The N.D represents that not obvious protein abundance difference was detected in the assays.

312 Differentially expressed proteins are involved in oxidation-reduction processes during cotton
313 fiber development [10]. During the fiber developmental transition, ascorbate peroxidase (APX)
314 family members, APX and APX6, and NADP-dependent D-sorbitol-6-phosphate dehydrogenase-like
315 protein (S6PDH) were found to be markedly upregulated in both cotton species. Alteration of fiber
316 cell reactive oxygen H_2O_2 levels in in-vitro ovule cultures has been reported to affect the
317 differentiation of the cotton fiber cell wall [26]. The increase in reactive oxygen (ROX) scavenging
318 enzymes APX and APX6 could maintain a low H_2O_2 level and regulate intracellular reactive oxygen
319 species homeostasis, thus indicating the importance in regulating H_2O_2 related signal pathways for
320 cotton fiber development Alternatively, the fiber cell elongation protein *Gh*fe1, as oxidation-
321 reduction related protein [27], appeared downregulated in both cottons, is consistence with the fiber
322 elongation gradually ceased during the transition. We also noted *Gh* cotton has upregulated the
323 peroxygenase 3 and the monodehydroascorbate reductase-like protein, as well as *Gb* specie has

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324 elevated expressions in the enoyl-[acyl-carrier-protein] reductase and the benzoquinone reductase,
325 suggesting that regulated oxidation-reduction pathways have specie-specific factors in rapid
326 transition.
327 Protein turnover is the net result of continuous synthesis and breakdown of body proteins and
328 ensures maintenance of optimally functioning proteins in organisms [28]. Protein synthesis, folding
329 and degradation pathways therefore are associated with protein turnover and amino acid
330 biosynthesis. When the major mass of the fiber becomes crystalline cellulose, the total protein content
331 of the developing cotton fiber eventually decreases during the fiber development process [10].
332 Among these identified proteins, the expression of 'protein degradation' class, including aspartic
333 protease-1 like, proteasome subunit beta type and carboxypetidase Y- like protein, are increased in
334 the development transition. Meanwhile, the 'protein synthesis' class that consisted of largely of
335 ribosomal proteins and the heat shock protein family were down-regulated in this stage, such as these
336 60S ribosomal protein L8-3-like; 40S ribosomal protein S14-3-like and 60S ribosomal protein L18a,
337 and 20 kDa chaperonin-like underwent obvious downregulation in both species. This turnover
338 regulation would allow plants to balance protein synthesis and degradation during fiber
339 developmental transition.
340 Fiber cell morphology is largely determined by the highly dynamic cytoskeleton architecture. In
341 this distinct stage, several cytoskeletal-related proteins with a dramatic increase, including the β-
342 tubulin-3 (TUB-3), annexin (ANN) were found in both cotton species. Upregulation of cytoskeletal-
343 related proteins are essential for the fibers normal morphogenesis changes. Indeed, these ANN and
344 TUB-3 have been reported to participate in cytoskeleton dynamic assembling and maintenance [29,
345 30]. Interestingly, other two known actin dynamic regulators, profilin and acid cyclase-associated
346 protein (CAP) are differentially upregulated among two cotton species. Dynamically changed
347 profilin that binds actin cytoskeleton has been shown to be important for restructuring cell shape
348 [31]. However, we detected the increased profilin only in *Gh* but not in *Gb* fibers. Additionally, a
349 greater than 2-fold upregulation of another actin-binding protein CAP was found in *Gb* cotton.
350 Identification of these cytoskeletal related proteins in development transition further supported
351 reorganization of the actin cytoskeleton is an important scheme in controlling direction of cellulose
352 fibril deposition in the developmental process switching from elongation to secondary wall
353 deposition.
354 In particular, among these DEPs, it appears that Ca^{2+}-signaling pathways are involved in fiber
355 development transition. Calcium mediated signaling plays an important role in cell division and
356 differentiation including root hair elongation [32]. Preferential expression of calcium binding proteins
357 during fiber initiation and elongation stages have been reported in cotton [10]. Several highly up-
358 regulated Ca^{2+}- signaling pathway proteins, including calmodulin-7 and calreticulin-like, were
359 detected in the developmental transition for both cottons. A few recent reports have shown the
360 overexpression of Ca^{2+}- dependent protein kinase1 (CPK1) stimulates the onset of secondary wall
361 deposition [33]. The probable calcium-binding protein CML13 was preferentially upregulated in *Gh*
362 fibers, which might regulate Ca^{2+} homeostasis in the developing fiber cells.
363 Several other signaling transduction molecules were identified in cotton fiber development
364 transition. Among them, ERBB-3 binding protein 1 and 14-3-3-like protein 2 were commonly
365 downregulated in both species. It has reported that 14-3-3 proteins participated in regulation of fiber
366 initiation and elongation by modulating brassinosteroid signaling and overexpression promotes fiber
367 elongation in cotton [34]. Therefore, the downregulated 14-3-3 protein is plausible actor in the
368 developmental transition from fiber elongation to secondary cell wall synthesis.
369 Additionally, in the fiber developmental transition stage, 14-3-3 protein 6-like, fasciclin-like
370 arabinogalactan protein 1 and STS14 protein-like were only downregulated in *Gh*, whereas another
371 key signal transduction related molecule, Ras-related protein RABA1f-like, was downregulated in
372 *Gb*. Moreover, a sulfurtransferase is reported to be abundant in the plant cell wall and plasma
373 membrane, promoting cell differentiation [35]. In *Gh* cotton fibers, the upregulation of
374 sulfurtransferase as well as guanosine nucleotide diphosphate dissociation inhibitor (GDI) was
375 recorded. While another upregulated signal transduction molecule frasciclin-like arabinogalactan

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protein 1 (FLAs1) was found only in *Gb*. These results indicate that several species-specific signal transductions may take place during this process and altered expression of various signaling molecules underpin a complicated signal transduction network regulating many downstream biochemical events during the transition phase.

While cellulose synthesis is gradually prevails during the late stages of fiber development, many metabolic pathways that are active during fiber elongation are repressed. In this developmental transition, multiple secondary metabolism related proteins, including shikimate O-hydroxycinnamoyltransferase-like (HCTL), naringenin (NAR), anthocyanidin reductase-like (ANRL) and chalcone-flavanone isomerase family protein (CHI), were found to be significantly downregulated in both species. Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine which have a wide variety of functions both as structural and signaling molecules [36]. Plant HCTL enzyme participates in phenylpropanoid biosynthesis [37]. The marked downregulation in HCTL expression, consistent with prior reports that cotton fiber thickening is negatively related to phenylpropanoid content, suggest that the HTCL associated with phenylalanine metabolic pathways might play a key role in the fiber developmental transition process. Also representing the temporal shift in protein expression pattern are down-regulated flavonoid biosynthetic pathways. As Yoo et al. reported, carbon resources might be reallocated in developing cotton fibers [3]. In this proteomic analysis, three enzymes, NAR, ANRL and CHI, all involved in the flavonoid biosynthesis pathway [38], were downregulated throughout this development stage in both species, suggesting that carbon resources have been transferred away from flavonoid metabolism and might be coordinated to direct carbon flux into cellulose during this stage, thus highlighting that phenylpropanoid and flavonoid metabolism represents a novel pathway with potential for cotton fiber improvement.

3.6. Validation of differently expressed proteins by qRT-PCR

To examine whether the differences in abundance were consistent with the differences at the mRNA levels, qRT-PCR was used to analyze the transcripts encoding 12 proteins with differing expression, belonging to various functional categories, at least two time points during fiber development stages (15-19, 19-23, and 23-27 dpa).

The qRT-PCR analysis indicated that expression patterns of 9 mRNA abundance (75% of 12 proteins) were highly consistent with the protein data, while the remained 3 proteins (25%) were partially consistent (**Fig. 7**). Nevertheless, the transcript and protein levels of ANN and CHI conflicted at 19-23 dpa. As has been noted previously, mRNA levels do not always correlate well with the level of corresponding protein mainly because of post-transcriptional regulation mechanisms such as nuclear export and mRNA localization, transcript stability, translational regulation, and protein degradation [39]. These results confirmed the reliability of the proteomic analysis results and proteomic analysis is essential for identifying the final products responsible for different cellular functions. Proteomic analyses are crucial for providing accurate pictures of the regulatory networks of functional genes/proteins.

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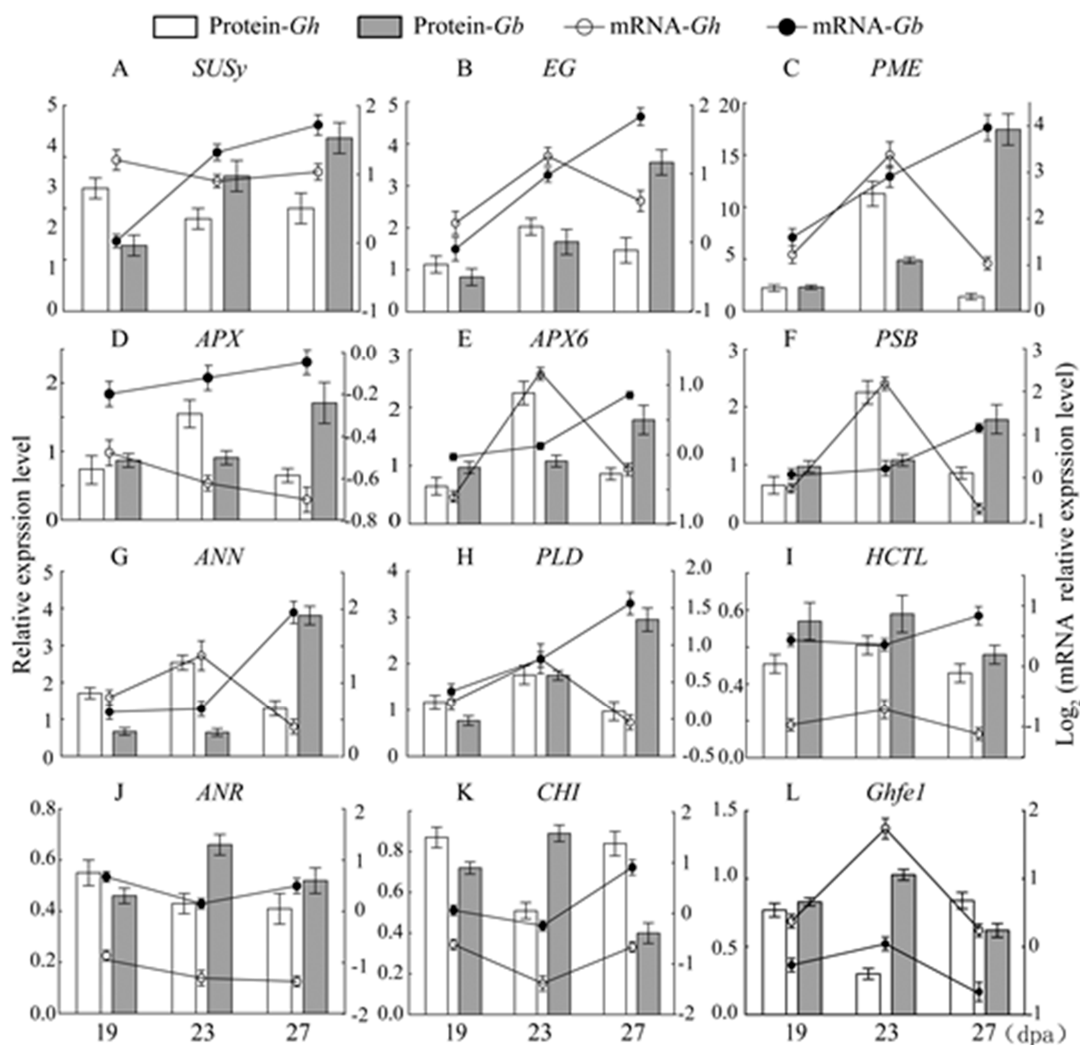
414



415 **Figure 7.** Validation of differentially expressed proteins by qRT-PCR. Cotton Ubiquitin7 gene was used as an
416 internal control. Error bars represent the standard deviation of the mean.

417 **4. Conclusion**

418 Cotton fiber development is a dynamic process that is accompanied by major regulation by
419 multiple and diverse protein expression networks and pathways. In present study, the analysis of
420 fiber morphological and proteomic dynamic across the development time course suggested a
421 significant variation at the timing of development transition and *Gb* indeed shares highly similar
422 development regulatory patterns to *Gh* during corresponding fiber development transition stage. The
423 profiling of protein expression dynamics have provided new insight into biological processes
424 governing fiber development in this distinct stage. Specifically, a number of species-specific proteins
425 with significant changed expression were also identified in cell wall transition. For instance, the
426 discrepancy in peroxidases and other signaling-related proteins, as well as *Gb* has higher
427 level upregulation in PME, SUS, CESA8 etc that would more significantly alter the composition of
428 pectin and cell wall polysaccharides. Further work can be beneficially directed toward understanding
429 connections within various regulated proteins and pathways in the cotton fiber development
430 transition.
431

432 **Supplementary Data**

433 Supplementary data is available online.

434

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441
442

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