Proteomics-based models of gene expression and cellular control of cotton fiber development

Running title:
Cotton fiber proteome dynamics: daily resolution

Keywords: G*ossypium hirsutum*, trichoblast, proteomics, machine learning, cellulose synthase, multi-omics

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Abstract

The cotton textile industry is based on the material properties of terminally differentiated trichoblasts. Tens of thousands emerge from the seed coat epidermis and march through a reproducible developmental program that includes interconversion between cell-autonomous and tissue-based morphogenesis. Proteomic analyses of purified fibers provide a way to associate molecules with cellular processes that directly affect morphological transitions and material properties of the harvested cells. However, insufficient temporal sampling and poor protein coverage have limited the extent to which proteomic data predict control mechanisms. Here we quantified the subcellular proteomes of purified fibers daily over a 20-day interval that includes diverse modes of elongation and cell wall remodeling. Thousands of reliable protein abundance profiles were assigned to expression groups and tested for associations with a broad array of phenotypes. Integration of proteomic and RNA-sequencing data revealed distinct modes of transcriptional and post-transcriptional control. Collectively these datasets provide large-scale gene function predictions that can serve as a toolkit to engineer cotton material properties.

Main

Cotton (*Gossypium spp*.) is the world's most important fiber crop and the foundation of a multi-billion dollar renewable textile industry [1,2]. Tens of thousands of unicellular seed coat trichoblasts synchronously differentiate and transform their length:width aspect ratios during development to ~3000:1 [3]. At maturity, each fiber contains a helically patterned cellulose-rich wall that desiccates after programmed cell death to generate harvestable thin, strong, spinnable fibers [4-6]. Fiber size, spinnability, and strength are the output of a morphogenetic program of anisotropic diffuse growth [7,8] with an often underestimated degree of complexity (Fig. 1). Phenotyping daily reveals an early tapering phase that is completed by 2 days post anthesis (DPA), during which the hemispherical fiber initial is converted to a thin elongated trichoblast [8,9]. After tapering the cell wall (CW) thickens as it enters a protracted phase of decelerating anisotropic diffuse growth [10,11], while simultaneously modulating plasmodesmata functions and turgor pressure [12]. Given progress in understanding the molecular and biomechanical control of trichoblast morphogenesis [8,13,14], there are emerging opportunities to use biotechnological interventions to engineer fiber traits. The goal of this research is to integrate high-temporal resolution quantitative proteomics, RNA-seq, and phenotyping to broadly predict cellular networks that mediate key developmental transitions.

A complexity of studying fiber cell "omics" is their coordinated development with the organ-scale growth and physiology of the enclosing capsule (colloquially termed a "boll") chamber (Fig. 1a,b and Supplementary Video 1). Fiber development also includes reversible transitions between cell-autonomous growth and tissue-morphogenesis during which the cells are tightly adhered [15]. Cotton has long served as a useful model for CW assembly and morphogenesis [16-18], and components of the molecular control continue to emerge [2]. A highly conserved cellulose patterning module underlies polarized diffuse growth in the plant kingdom [19], and the details of how interactions among cortical microtubules, patterned cellulose synthase (CESA) activity, and CW stress-sensing operate to control cell shape changes are beginning to emerge [20,21]. Strong lateral interactions between microfibrils [8,13,22] and interactions between fiber and matrix components generate domains of the CW that deform asymmetrically in response to turgor-generated mechanical stress in the CW [8,13,22]. Spatially patterned cortical microtubules influence microfibril deposition throughout development to maintain cell diameter and pattern a helical secondary CW (SCW) [4,8,11,16]. Important cotton microtubule-associated proteins are being discovered using forward and reverse genetic approaches [23-25]. The microtubule-CESA module does not function in isolation; actin-based intracellular transport, vesicle trafficking, metabolism and redox control, and hormone signaling function in concert during fiber development [2].

Proteomic profiling provides one way to obtain direct measurements of the protein machineries and post-translational controls that enable effective morphogenetic work and coordinate diverse functions, some of which reside in different subcellular compartments. Historically, proteomic analyses of cotton fibers have identified proteins with altered abundances as a function of domestication [26-28], hormone or inhibitor responses [29,30], and developmental stages [31-33]. In general, the number of discovered proteins is low and the temporal sampling of fiber development is limited to 5-day intervals at best. Gel-free workflows and increasingly sensitive instrumentation enable the quantification of thousands, rather than dozens, of proteins in a typical experiment [29,34,35]. This presents an opportunity to gain broad insights into the protein dynamics that underlie the numerous developmental transitions during fiber development that are not known.

Here we combined daily sampling and simple cell fractionation procedures to analyze how protein abundances change in distinct subcellular compartments during fiber development. Our analytical

pipeline allowed us to generate thousands of reliable protein abundance profiles spanning a broad period of fiber development, encompassing primary cell wall synthesis and the transition into secondary wall synthesis. Clustering methods identified unique protein expression groups that reflected unique activities of the apoplast. Multiple groups had clear temporal transitions and broad memberships across the cell fractions. Correlation analysis with phenotypic variability across the same timeline provides a way to predict suites of genes that coordinate the unidirectional progression of fiber development. Integration of RNA-seq and proteomic data can provide large-scale predictions about general modes of gene expression control [36,37]. We used combinations of cross-correlation analysis and machine learning methods to globally predict modes of transcriptional and post-transcriptional control. Collectively these data and analytical methods provide new knowledge about evolutionarily conserved proteins and cellular processes that control morphology in the plant kingdom. These data resources also provide a new way to link cellular processes with important phenotypes and predict groups of gene products that mediate key developmental transitions.

Results

Protein profiling of developing cotton fiber by proteomics

A central goal was to generate reliable time-series data of protein abundance in multiple subcellular fractions from fiber tissue. Methods to isolate highly purified fiber samples and generate high-quality cell fractions with limited cross-contamination with broken cells were developed [38]. The method enables precursor ion-based quantification of a diverse population of proteins within and between cell fractions. Here we scaled up the procedure to include multiple locules from a single boll to increase coverage of the crude cytosolic (S200) fraction (Fig. 1c) and included three replicates daily from 5-24 DPA. The entire developmental progression, from anthesis at 0 DPA to the show-stopping event of boll opening at 48 DPA, is shown in Fig. 1a,b and Supplementary Video 1. Protein yields in the different cell fractions are included in Supplementary Data 1. Our approach was to conduct quantitative shotgun proteomics of cytosol, microsome, and extracellular proteins obtained from dissected locules and purified fibers at daily intervals from 5 to 24 DPA (Fig. 1c). The earliest timepoint was chosen based on the minimum requirements for adequate protein precipitation from the soluble fractions, and the final time point was selected in order to capture a complete transition to SCW synthesis.

A crude apoplast (APO_T) fraction was generated after careful dissection and brief immersion of the intact locules in the isolation buffer. This protocol minimizes contamination from broken cells and has been shown to generate a distinct apoplast fraction with unique protein multimerization behaviors and composition compared to cytosol and microsome fractions [38]. After immersion, fibers were manually dissected, homogenized, and subjected to differential centrifugation to generate a crude microsomal (P200) and a soluble (S200) fraction (Fig. 1c). Across the 5 to 24 DPA window, 4316 proteins were reproducibly identified and quantified in 2 of 3 biological replicates of at least one cell fraction, providing the most comprehensive cotton fiber proteome coverage to date (Fig. 1d and Supplementary Data 3). Cotton (*G. hirsutum*) is an allopolyploid with two subgenomes (A, D), and as such the duplicated genes (homoeologs) are encoded by the A- and D-subgenomes, such that they potentially can be distinguished based on diverged peptide sequences [34,35]. Diagnostic homoeolog-specific peptides were detected for ~55% of the cases and these proteins are annotated with "A" and "D" suffixes following the Locus ID (Supplementary Data 3). The P200 fraction consistently had the most diverse protein populations over the time course, and, as observed previously [38], was more similar to the APO_T compared to S200

(Supplementary Data 3c). The APO_T fraction primarily consists of membrane-enclosed particles with a highly heterogeneous composition [38]. Widespread overlap among the different cell fractions can occur because many proteins are dually localized [39]. Our pipeline generated protein quantification across over 6 orders of magnitude (Fig. 1e). The protein types amenable to proteomic analyses are extremely diverse (Supplementary Data 3). The coverage of metabolism in this dataset is deep (Extended Data Fig. 1) and provides broad experimental evidence for the specific gene products that operate in central metabolism, phenylpropanoid biosynthesis [29,40], fatty acid synthesis [41], and cell wall assembly [42].

A protein was retained for abundance profiling across the time course if it had non-zero values in three or more fractions with at least two of them on 2 consecutive days. Proteins that met this detection frequency threshold tended to have slightly higher signal intensities in each cell fraction compared to those that did not meet the threshold (Extended Data Fig. 5). However, the protein population composed of those detected in at least 2 of 3 consecutive DPAs and the subset detected in 10 or more DPAs had similar intensity distributions (Fig. 1e and Extended Data Fig. 5), indicating that signal intensity is not the primary determinant of expression profile variability. The raw and normalized intensity data and plotted distributions are provided in Supplementary Data 3 and Extended Data Figs. 2–4.

Our deep proteome profiling provides a glimpse into compartmentalized protein behaviors during development, identifying diverse groups of proteins that coordinate fiber development. However, the widespread occurrence of missing values is a widely recognized shortcoming of label-free quantitative proteomics. We developed interpolation and data filtering methods to identify informative expression profiles in the three subcellular locales. To identify expression groups, potentially informative profiles were analyzed using a machine learning-based clustering method termed Self-Organizing Map (SOM) analysis [43,44]. Each SOM group has a “code” that reflects a central expression pattern of the members (Fig. 2a and Supplementary Data 4). A group was scored as unreliable if: 1) the code profile was punctuated by missing values across the timeline; 2) the profile pattern reflected variability in protein coverage in specific DPAs (Supplementary Data 3d); or 3) the pattern was defined by strings of 3 adjacent zeros that could simply reflect random gaps in the data that were not filled by interpolation. Following this filtering, 39 groups were classified as unreliable, and 44 potentially informative groups were filtered further to remove unreliable members with discordant features in their expression profiles (Fig. 2b, Extended Data Figs. 2–5). In the end, these 44 expression groups provide large-scale functional data on gene sets and cellular activities (Fig. 2c, Supplementary Data 5). These data provide clues about gene functions that may operate during specific developmental windows in particular subcellular locales.

Many of the profiles were non-uniform, with sharp abundance transitions often reflecting a shift to or from consecutive days of non-detection. Those classified as “increasing” had a clearly defined timepoint at which the protein transitioned from below to above the detection limit (Fig. 2c). The inverse was true for the profiles classified as “decreasing”. We hypothesize that these clearly varying expression patterns reflect rearrangements of suites of cellular machineries as the fiber population undergoes developmental transitions. Some non-uniform expression groups were defined by a specific subcellular compartment. Gene ontology (GO) enrichment analysis shows that P200 protein expression groups with an increasing pattern have hydrolase activity, guanyl nucleotide binding, and catalytic activity terms, whereas S200 was enriched in response to stress term (Supplementary Data 6). Constitutively expressed proteins classified as non-uniform were flagged if there was at least a 4-fold increase or decrease in the mean protein abundance of the group members during development (Supplementary Data 5). These “constitutive increase/decrease” groups may reflect broad reprogramming of gene expression relative to the transition to SCW synthesis [45] and/or proteins with unequal activities in the elongation and SCW

phases. A large number of proteins had flat relative abundances and were classified as uniform. These proteins may define part of the "constitutivome" that includes essential functions that operate across the developmental phases.

Integration of protein dynamics modules with fiber phenotypes

We next wanted to correlate protein expression profiles with phenotypic transitions. To generate a merged set of profiles aggregating protein abundance dynamics across the cell fractions, we turned again to SOM. The cell fraction profiles were merged to generate a protein expression model that included 11 merged groups with distinct non-uniform profiles (Fig. 3a and Supplementary Data 7). The mean normalized intensities of the merged group members were used to estimate the behavior of the group. Aggregation of 7 developmentally variable fiber phenotypes from the same or similar developmental windows [11,46] provided a way to test for significant associations between protein expression groups and phenotypic transitions using correlation analyses (Fig. 3c, Supplementary Data 8).

The M16 group was most strongly positively correlated with the transition to SCW synthesis, which is characterized by transitions in cellulose microfibril angle, increases in cell wall thickness, and an increasing proportion of cellulose in the cell wall (Fig. 3c and Supplementary Data 8b). M16 contained proteins present in all three subcellular compartments and included many proteins involved in CW biosynthesis, as well as many previously uncharacterized proteins (Fig. 3d and Supplementary Data 8 and 9). M16 also contained *G. hirsutum* orthologs of each of the SCW-associated CESAs [45,47] that were induced at ~16 DPA to similar levels (Fig. 4d and Supplementary data 9a). M19 was negatively correlated with SCW transition and may contain proteins that are specifically involved in the fiber elongation and remodeling of the primary CW (Fig. 3c and Supplementary data 7c and 8b). M14 was also negatively correlated with SCW transition, but this group was composed solely of APO_T members. By comparing these data with those in a purified cotton EV dataset [38], we found that M14 members were either specific to the particulate fraction or dual-localized in the soluble and particulate fractions of the apoplast (Supplementary data 7d). M14 contained more than half of the total APO_T proteome. The particulate fraction was enriched in spherical stain-excluding compartments that could be visualized using negative staining and transmission electron microscopy (Fig. 3b). To determine if the apoplastic particles (APO_{p200}) had a unique polysaccharide composition, their glycome profiles were compared to those of the P200 and APO_{s200} fractions. Several epitopes related to xylan and xyloglucan were specifically enriched in the APO_{p200} particles compared to the other cell fractions and many other epitopes were selectively depleted (Supplementary Data 10). These data provide further evidence for their unique composition and may reflect their mode of generation. M9 and M18 were also APO_T-specific expression groups with similar profiles (Fig. 3c). These profiles may reflect the developmentally regulated biogenesis of extracellular vesicles.

M20 declined from maximal levels at 5 DPA and was most strongly correlated with the decelerating relative growth rate and a reduced proportion of pectin (Fig. 3c). M20 may contain groups of proteins associated with a fiber state with a faster relative growth rate. M11 and M17 were anti-correlated with relative growth rate and auxin-response factors, ABA receptors, and vesicle trafficking machineries (Supplementary Data 7c, column T, M11) that enable persistent axial growth and/or early components that prime the cell for the transition to tissue-based morphogenesis [15] or SCW synthesis [42]. Many of the merged protein groups correlated with phenotypic profiles, and these analyses provide models of how diverse types of gene products function in multiple cellular locations to coordinate developmental transitions.

Integration of mRNA and protein dynamics: distinct modes of gene expression control

To globally analyze potential modes of gene expression control, we integrated the proteomic profile data with fiber transcript data obtained using the same conditions and developmental time points [45]. Pearson correlation coefficients (PCC) were calculated for 6809 genes in which protein profiles could be matched to a single homoeolog (see methods, case 1) or a pair of homoeologs (Fig 4a and Extended Data Figs. 10-12). We initially focused on proteins with a non-uniform expression pattern, i.e., the mRNA/protein correlations in the "increasing" or "decreasing" categories that were stronger in the P200 and S200 fractions than APO_T fraction (Fig. 4b, Supplementary Data 12). About ~ 48 % of mRNA/protein pairs in P200 and S200 had PCC values > 0.75, and protein abundance could be explained by transcriptional control. In general, the APO_T proteins in both expression groups were weakly correlated with their mRNAs because this cell fraction was dominated by putative extracellular vesicle proteins that may have reduced abundance after ~20 DPA (Fig. 3a). In addition to the APO_T groups, those showing a negative correlation could be explained by the spatial and functional compartmentalization of gene expression regulation, highlighting the importance of subcellular localization information to understand mRNA-protein correlations. However, there was evidence for transcriptionally regulated proteins in the apoplast. A cotton ortholog of secreted signaling molecule STOMAGEN, usually associated with non-cell-autonomous cell proliferation [48,49], was downregulated at ~14 DPA and correlated with transcript levels (Supplementary Data 7).

To conduct a more open-ended test for commonly observed or non-linear patterns of gene expression control, a weighted SOM clustering was conducted by combining the mRNA and protein data for 1228 different P200 proteins with informative profiles (Fig. 4c and Supplementary Data 13). The full clustering result is shown graphically in Extended Data Fig. 13. The analysis produced three groups (V8-V10) with a total of 48 members with coherently increasing protein and mRNA expression, and three groups (V81,91,92) with a total of 50 members with coherently decreasing expression (Fig. 4c), of which 48 of 98 were captured in the PCC analysis described in Fig. 4b. Among the large population of proteins with a more uniform expression, there were 79 genes in which mRNA levels fell by at least 8-fold over the time course (Fig. 4c). These examples could reflect post-transcriptional controls in which the mRNA translation efficiency increases or protein stability increases during development. In V30, transcript levels increased 8-fold, but protein levels were stable (Fig. 3c), indicating that translation of the mRNA may be regulated or the steady state of the protein may be the result of physical interactions with a stabilizing factor. The SOM-weighted cross-correlation analyses provide quantitative information about how diverse types and timings of gene expression control operate during fiber development.

To partially validate our analyses with knowns, we queried our list of genes predicted to be subject to temporal transcriptional control with those known to be involved in CW assembly or the transition to SCW synthesis (Supplementary Data 14). For example, among the 67 proteins present in the M16 module, 44 were predicted to be transcriptionally regulated. Several orthologs of documented players in SCW synthesis (e.g., cell wall CESAs, COBRAs, and WAVE-DAMPENED-LIKE2 proteins) were reliably detected in the P200 fraction from fiber cell extracts (Fig. 4d). GhCESA4-B, 7-A/B, and 8-B proteins were first detected with a ~2-day lag compared to when the corresponding mRNAs first increased. A database of cotton orthologs to predicted Arabidopsis cell wall genes (Cell Wall Genomics database; https://cellwall.genomics.purdue.edu/) was created, and a subset of those were present in the set of predicted transcriptionally controlled genes (Supplementary Data 14). These data provide a glimpse

into the regulatory complexities of fiber development and a source of promoters and gene products that appear to be subject to transcriptional control and more easily modulated in transformed lines.

Discussion

This paper leverages sensitive and accurate quantitative proteomics with the synchronized process of cotton fiber morphogenesis to broadly predict protein networks that orchestrate fiber development. The data are derived from a single cell type that develops in the insulated environment of the developing capsule. Daily sampling revealed an unexpected complexity: an extended phase of growth deceleration in early development, followed by waves of gene activation and suppression that precede the transition to SCW synthesis and multiple phases of cellulose microfibril and cell wall matrix remodeling [11,38,45,46]. These transitions are not simply anchored to a general transition to SCW synthesis. Our proteomic dataset provides a conceptual model of how networks of proteins in multiple subcellular locations coordinate cellular functions and fiber phenotypes during development.

The proteomic resources generated here provide experimental support for over 4,300 fiber proteins, and reliable abundance profiles over a wide window of development for more than 2,900. This dataset provides useful time-series data on the localization and abundance of a functionally diverse population of enzymes, hormone signaling proteins like auxin response factors and ABA-receptors, as well as proteins that span the full breadth of molecular functions in the cell. There are many enzymes that are specifically associated with fiber elongation, including those involved in flavonoid, anthocyanin, and wogonin metabolism (Extended Data Fig.1 and Supplementary Data 5 and 6). The roles of these small molecules in fiber morphogenesis merit further analyses. All of the proteomic and associated RNA-seq data are publicly available in a simple search interface (link to be provided).

The SOM clustering method and cross-correlation with time-resolved phenotypes were used to generate models of how protein groups in multiple cellular compartments are linked to specific developmental transitions (Fig. 3). The merged profiles captured constitutively expressed proteins with uniform (M2,M3,M4,M5), increasing (M6,M7), and decreasing (M9,M10) abundances across the timeline. These proteins likely provide essential functions that may be more likely to be subject to post-translational controls. Many proteins were assigned to groups with clear time points of abundance change (M11,M14,M16-M20). This approach enabled us to identify groups of up- and down-trending proteins that transition at a similar time point. There were expression group pairs that were most strongly associated with growth rate variability (M20,M17) and others with the transition to SCW synthesis (M16,M19). Gene knockout experiments will be needed to determine how accurately these gene products are linked to these specific developmental transitions.

In the case of the transition to SCW synthesis, many key players are conserved across species and cell types with a thickened SCW [50]. Our dataset contains orthologs of each of the three CESA clades [51] and COBRA-like [52] proteins that are known to influence SCW synthesis in cotton, and many of them appear to be transcriptionally regulated (Figs. 3d and 4d). SCW patterning is a complex process that includes a radical reorganization of the cytoskeletal, vesicle trafficking, and cell wall assembly systems [53]. This proteomics analysis provides new insights into the particular CESAs and systems of proteins that operate prior to (M19) and after (M16) the transition to SCW synthesis. Some of expression groups may reflect reversible transitions in cell-cell adhesion as fibers interconvert between cell-autonomous and tissue-like morphogenesis at ~5 and ~20 DPA [15]. These different modes of growth may partially explain relative growth rate deceleration and the functional roles of protein expression groups correlated with

growth rate control (Fig. 3c). A prior publication on the apoplast proteome [38] and the time-series analyses here point to the existence of heterogeneously sized membrane-enclosed compartments in the extracellular space (Fig. 3b). The merged expression group profiles M18 and M14 reflect time points of transition to and from tissue-based morphogenesis (Fig. 3a). We speculate that these vesicle-enclosed compartments cannot simply diffuse from the plasma membrane to the extracellular space but are generated when cell-to-cell contact structures that include cytoplasmic components experience nanoscale mechanical failure as bundled fibers grow at different rates or directions.

Integration of proteomic and RNA-seq data can reveal distinct modes of gene expression control. These data also define novel temporal relationships between transcriptional upregulation, protein detection, and associated phenotypes (e.g., increases in cellulose content at 17 DPA). We identified a large set of transcriptionally activated and repressed genes at different stages of development (Fig. 4b,c). These lists are likely reliable as they identified specific *G. hirsutum* loci encoding orthologs of known players [25,51,52] as well as a broader set of unknown genes that have been annotated to have CW-related functions (Fig. 4d). These gene sets provide a potential toolkit of promoters and genes that may be effectively used to modulate fiber traits. Other potentially useful gene sets with constitutive expression or discordant transcript and protein profiles likely reflect unknown post-translational control mechanisms and will require further research to develop methods to alter their activities.

Conclusions and future challenges

Fiber development is an experimentally tractable system that has enormous potential in the context of architectural engineering for enhanced fiber traits. This potentially is likely to be increased by future improvements to proteomic pipelines. Only ~10% of the expressed genes were detected here (Fig. 4a), and there are too many missing values in the daily data matrix. In this respect, improved instrumentation and organelle separation methods would facilitate new insights into the overall proteomic composition and transformation states during cotton fiber development. Finite element biomechanical models of growing trichoblasts predict key CW material properties and CW geometry/force-sensing mechanisms underlie morphogenesis [8,21]. Discovery of key controlling genes can be accelerated by finding better ways to integrate time-series mRNA, proteome, and glycome data with genetic maps and gene function predictions. The barriers to cotton fiber transformation are diminished, so it is now possible to validate these predictions at a reasonable scale [54]. These improvements increase the prospects for crop enhancement via cotton fiber engineering. We anticipate growing proteomic datasets and additional high-dimensional transcriptomic and metabolomic data [40] will be fully aggregated in public databases [55,56] to fuel machine learning and AI-driven approaches that broadly predict relationships among genetic maps, molecular features, cellular functions, and important fiber traits.

Figures

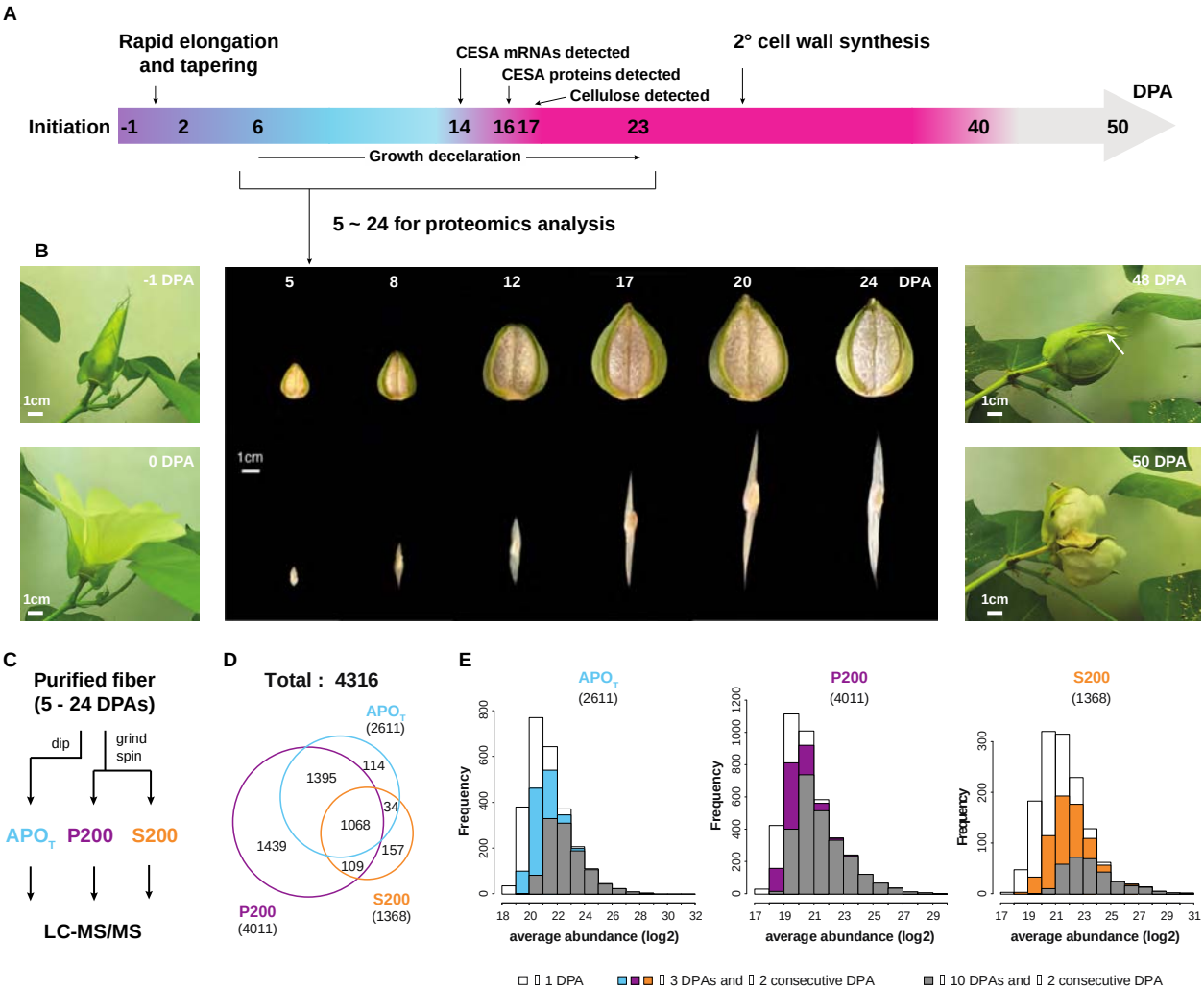


Fig. 1. Deep proteome profiling across fiber development in cotton (*G. hirsutum*) plants: cell fractionation and quantitative proteomics.

(A) Key developmental stages of cotton fibers [11,45,46]. Flower opening day is defined as 0 days post anthesis (DPA).

(B) Phenotypes of flower, dissected bolls, and combed fibers at selected time points for proteomics analysis (5 to 24 DPAs). The white arrow indicates the opening of the boll at 48 DPA. A sepal was removed from the flower for visualization purpose. A movie of the cotton flower and boll development is available in Supplementary Video 1.

(C) Cell fractionation of triplicate samples for quantitative proteomics analysis [38].

(D) Proteome coverages for the three different subcellular compartments.

(E) Distributions of average protein abundances across DPAs showing missing values.

APO_T: apoplast; P200: crude microsome; and S200: soluble fractions. Parentheses show numbers of proteins in each cellular proteome.

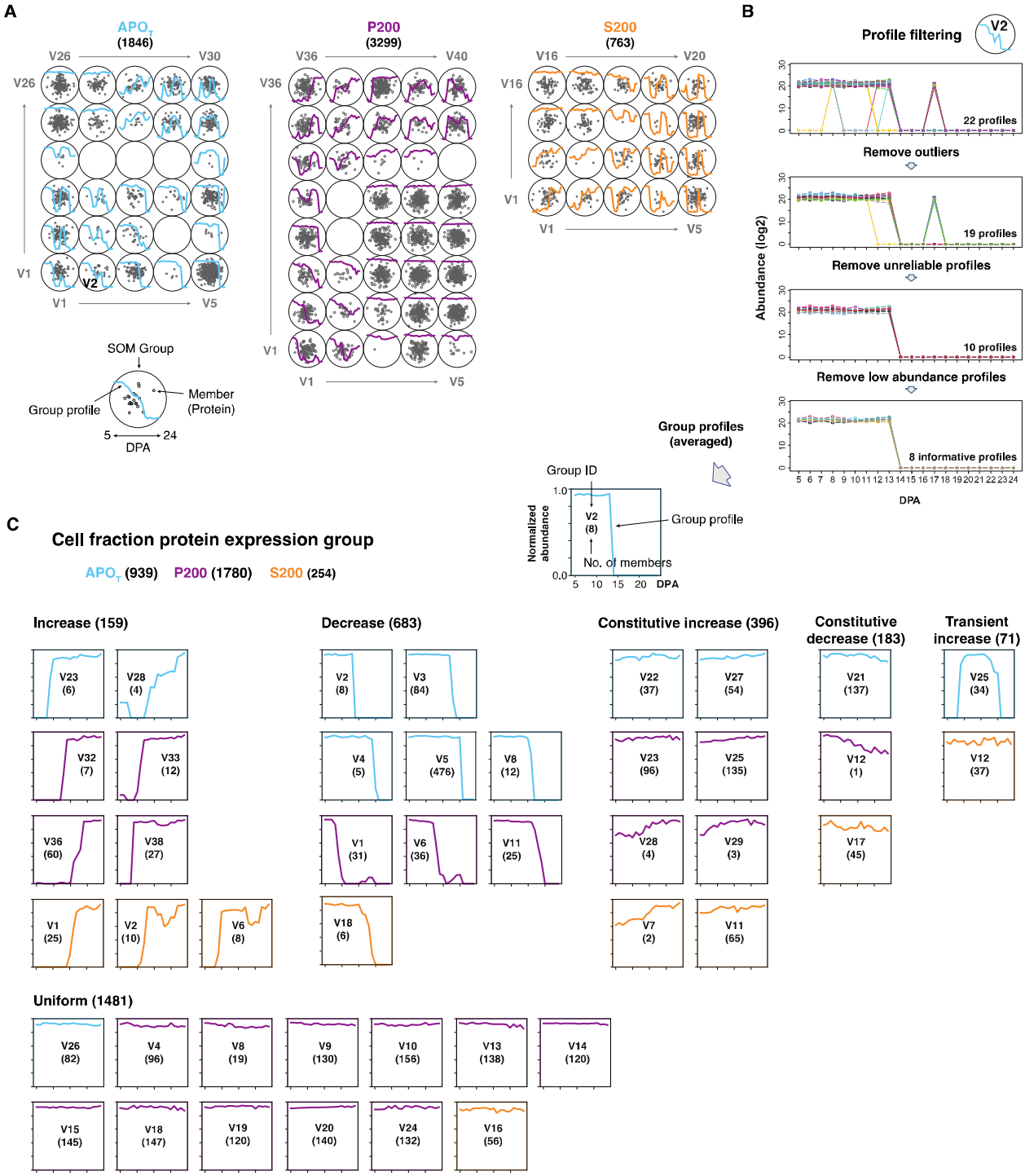


Fig. 2. Generating a core set of protein expression modules during developing cotton fibers.

(A) Grouping of similar protein profiles in the three different subcellular proteomes using a machine learning-based self-organizing map (SOM) approach.

(B) Data filtering to identify the subset of reliable members of an expression group. APO_T V2 was used as an example. Outliers were removed using pair-wise similarity with group representing profiles (Cosine similarity < 0.8). Unreliable profiles, which refer to isolated non-zeros, were moved from the group. Then, lower-abundance proteins were filtered out to create protein groups representing profiles.

(C) A set of informative protein expression groups across fiber development in the three different cellular fractions.

APO_T: apoplast; P200: crude microsome; and S200: soluble fractions. Parentheses show numbers of proteins in each cellular proteome or each group.

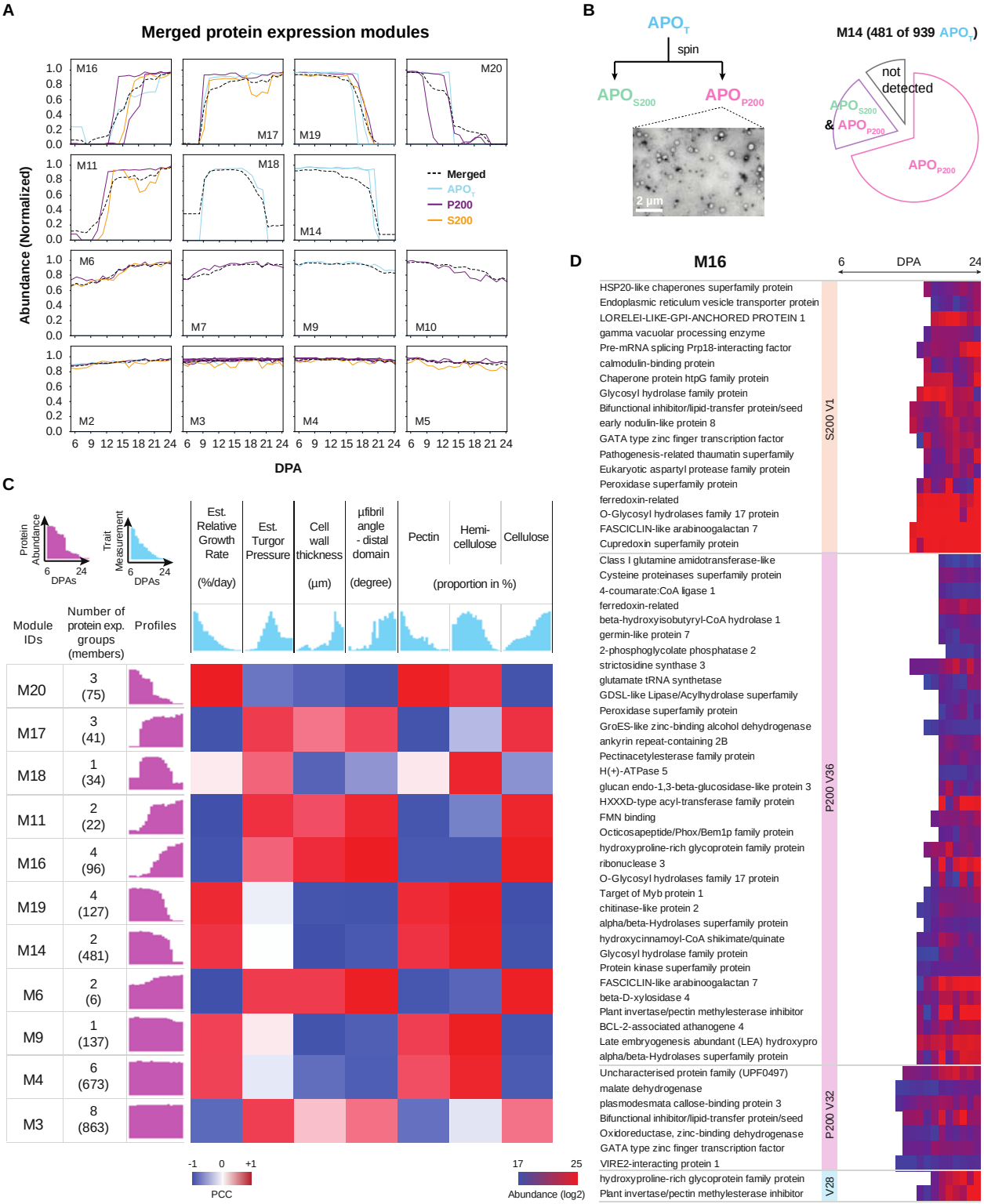


Fig. 3. Integration of protein dynamics modules with fiber phenotypes.

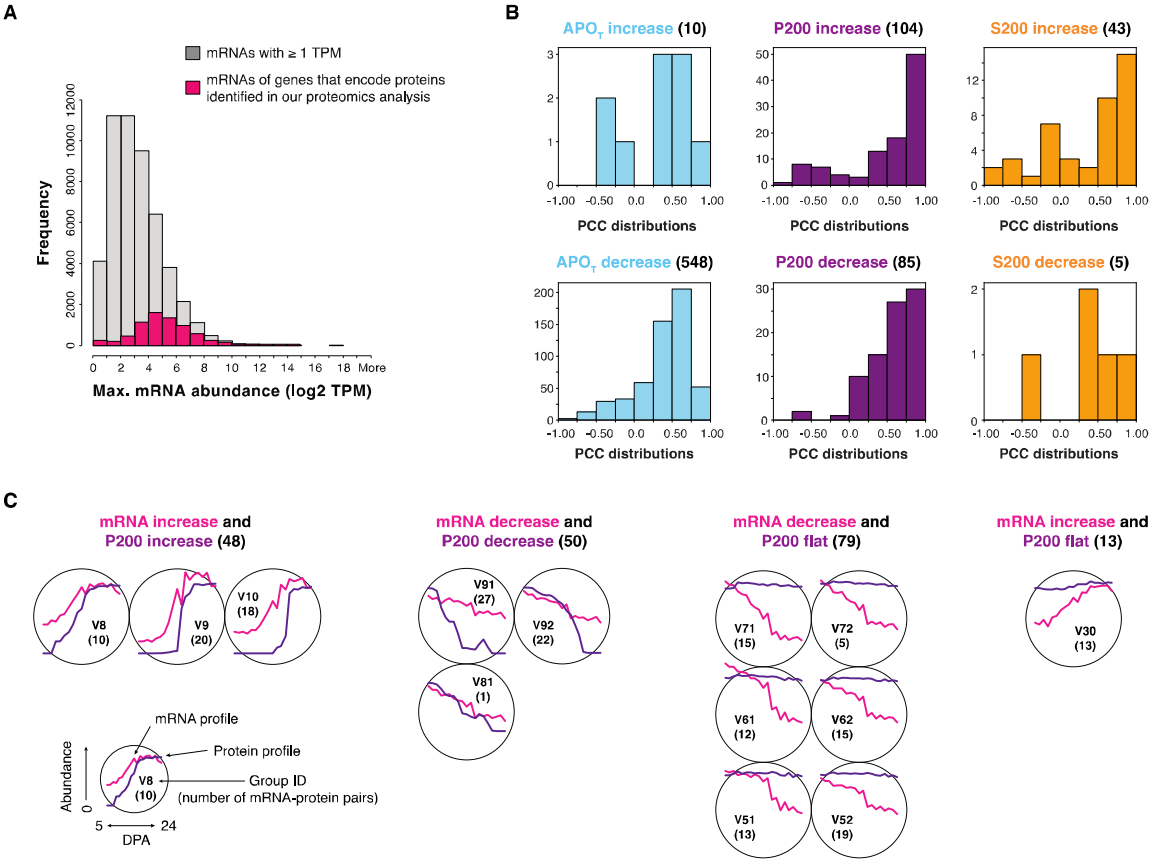
(A) Protein expression modules merged from the APO_T, P200, and S200 cellular fraction protein expression groups.

(B) M14 protein expression group reflects developmentally regulated extracellular vesicles. The proteomics pipeline used to identify APO_{P200} and APO_{S200} proteins [38]. TEM images of vesicles present in APO_{P200}. Overlap of APO_T proteins in M14 with APO_{P200} proteins (Supplementary Data 7d).

(C) Integration analyses of fiber phenotypes and protein network modules. Pearson correlation coefficient (PCC) between protein patterns and phenotypes [11,46] was calculated. Module profiles show the merged protein expression profiles.

(D) M16 protein expression module shows similar and unique compartment specific profiles that are associated with cellulose content (proportional) increase across development. A full list of proteins in the M16 can be found in Supplementary Data 9a.

APO_T: apoplast; P200: crude microsome; and S200: soluble fractions. Parentheses show numbers of proteins in each cellular proteome or each group.



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Clade	Protein name	Gene ID
CESA 4	GhCESA4-B-At	Gohir.A08G045800
	GhCESA4-B-Dt	Gohir.D08G056300
CESA 7	GhCESA7-A-At	Gohir.A07G037800
	GhCESA7-A-Dt	Gohir.D07G041900
	GhCESA7-B-At	Gohir.A05G008700
	GhCESA7-B-Dt	Gohir.D05G009400
CESA 8	GhCESA8-A-At	
	GhCESA8-A-Dt	Gohir.D05G156700; Gohir.D05G156801
	GhCESA8-B-At	Gohir.A10G035300
CESA Interacting	GhCOBL4-D-At	Gohir.A11G159400
	GhCOBL4-D-Dt	Gohir.D11G165500
Microtubule associated proteins (MAPs)	GhMAP65-8-Dt	Gohir.D02G150100
	GhWD2L-At	Gohir.A10G113800
	GhWD2L-Dt	Gohir.D10G153300
	GhTPX2-At	Gohir.A11G299000
	GhTPX2-Dt	Gohir.D11G313800
	GhIQ domain 32-At	Gohir.A05G188000
Cell wall biogenesis	GhGH17-At	Gohir.A04G012484
	GhFLA7-Dt	Gohir.D08G059800
	GhLPTL-At	Gohir.A02G033100
	GhPMEI-Dt	Gohir.D11G084500
	GhPMEI-At	Gohir.A11G079800
	GhRAB11A-Dt	Gohir.D08G012300
	GhRABA1C-At	Gohir.A10G034800
	GhRABA1C-Dt	Gohir.D10G035500
	GhFLA11-Dt	Gohir.D11G343800
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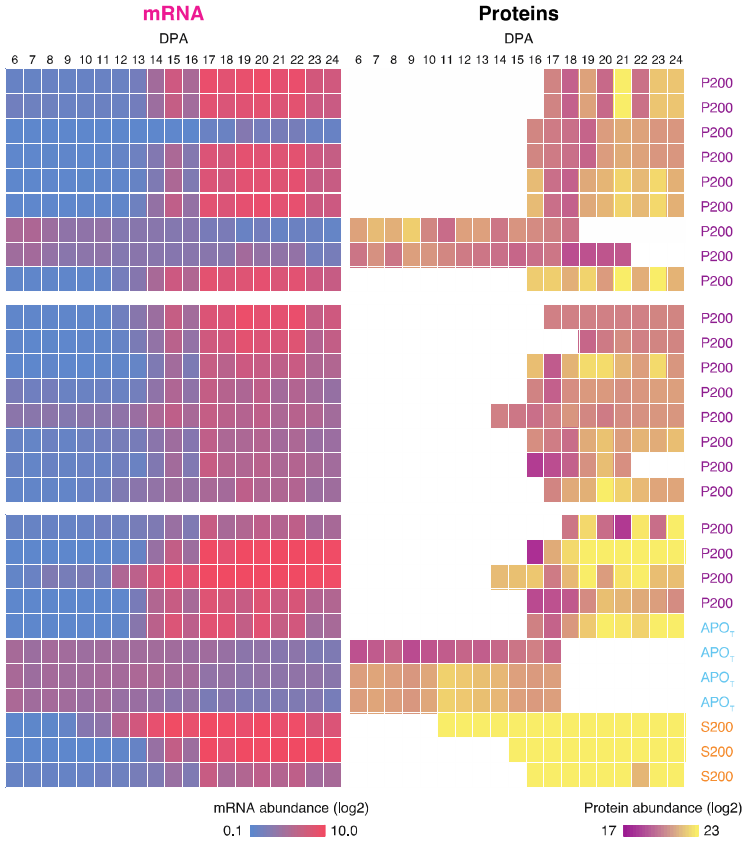

Fig. 4. Global analysis transcript and protein profiles across fiber development.

(A) Coverages of proteins present in the cell fraction protein expression groups over the cotton fiber transcriptome data [45]. Maximum mRNA and protein abundances across fiber development were visualized. The filtered transcriptome dataset is available in Supplementary Data 11.

(B) Pearson correlation coefficient (PCC) between mRNAs and proteins (Case 1 proteins; please see the methods) present in the protein expression groups with up or down profiles in Fig. 2c.

(C) SOM-based weighted cross-correlation of paired mRNA and proteins that have flat profiles across development. Protein patterns were defined in Fig. 2c, and mRNA patterns were defined using fold change (8-times abundance change). The entire SOM result can be found in Supplementary Data 13 and Extended Data Fig. 13.

(D) Transcriptionally controlled known protein markers that function in secondary cell wall synthesis.

Example mRNA and protein abundance profiles of transcriptionally regulated genes present both in the M16 module and assigned to the V8, V9, V10, and V92 groups of the weighted cross-correlation SOM in Fig. 4c. A broader list of the predicted cell-wall transcriptionally regulated dataset of the abundance profiles of proteins across fiber development and PCC values are provided in Supplementary Data 14. At and Dt indicate homoeologs for the A and D genomes.

APO_T: apoplast; P200: crude microsome; and S200: soluble fractions.

Online Methods

Cotton growth conditions

Gossypium hirsutum cv. TM1 plants were cultivated in a Conviron® E15 growth chamber (Conviron, Pembina, ND, USA) at the Plant Growth Center in Purdue University as previously described [38]. Seeds were sown in a 3 gallon pot with a soil mixture composition prepared as 4:2:2:1 soil:perlite:bark:chicken grit. The growth chamber was controlled to retain 50-70 % of relative humidity and remain with a day/night setting of 28/23 °C and 16/8 h at a light intensity of 500 μmol m^{-2} s^{-1} (fluorescent lamps of 28 Sylvania F72T12/CW/VHO 100W and 4 Sylvania F24T12/CW/HO 35W; incandescent bulbs of GE 60W light 130V A19). The 30 mins two-step ramp-up and 30 mins two-step ramp-down periods (15 minutes at 166 μmol photons + 15 minutes at 336 μmol photons) were programmed at the daytime beginning and the ending, respectively. At anthesis, flowers were marked as 0 day post anthesis (0 DPA). Three bolls were collected daily from 5 to 24 DPA for a total of 60 samples (20 days × 3 replicates). The harvested cotton bolls were immediately maintained on ice and carefully dissected to get four intact ovules from each boll.

Cell fractionation into apoplast (APO_T), microsome (P200), and cytosolic (S200) fractions

The APO_T, P200, and S200 fractions were prepared as previously described [38]. One intact ovule (700 mg) in each locule was dipped in 5 mL of pre-chilled microsome isolation buffer (MIB) [50 mM Hepes/KOH (pH 7.5), 250 mM sorbitol, 50 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 2 mM PMSF and 1% (v/v) protein inhibitor cocktail (160 mg/mL benzamidine-HCl, 100 mg/mL leupeptin, 12 mg/mL phenanthroline, 0.1 mg/mL aprotinin, and 0.1 mg/mL pepstatin A)]. After 10 minutes of incubation under gentle shaking, the resulting buffer solution was recovered through 2 layers of cheesecloth. Then, the entire APO_T fraction was used for protein precipitation using an acetone precipitation method.

For P200 and S200 fractions, fibers were directly isolated from the ovule material used for the above APO_T collection [38]. Purified fresh fiber tissues were homogenized under the cold MIB (sample to MIB ratio was 1:4) using Polytron homogenizer (Brinkman Instruments, New York, NY, USA) with chilled blade tip with 10 seconds grinding, 1 minute rest on ice, and another 10 seconds grinding. The homogenate was filtered with 4 layers of cheesecloth pre-soaked in cold MIB, and the cheesecloth was further squeezed to get a residual sample in the cheesecloth. Then the filtered homogenate was further centrifuged on an Allegra X-30R centrifuge (Beckman Coulter Life Sciences, Indianapolis, IN, USA) at 1,000 x g for 10 min to remove debris. The supernatant was fractionated at 200 k x g for 20 minutes at 4°C using a Beckman Optima Ultracentrifuge with TLA110 rotor (Beckman Coulter Life Sciences, Indianapolis, IN, USA) into a cytosolic (S200) fraction and pellet. The resulting pellet was washed with cold MIB, incubated on ice for 10 minutes, and ultracentrifuged at the same condition as shown above, and the washing step was repeated. The pellet was defined as a microsome fraction (P200) and directly subjected to protein precipitation using an acetone precipitation method. Protein yield information for the three cell fractions is provided in Supplementary Data 1.

Determination of protein concentration

For the APO_T and S200 cellular fractions, dried pellets in the acetone precipitation were dissolved and denatured in 100 μL of 8 M Urea for 1 hour at room temperature. For the P200 fraction, 200 μL of 8 Urea was directly added over the final pellet and incubated for 1 hour at room temperature to denature

proteins from membranes. Undissolved debris was removed by centrifugation at 12 k x g for 15 min using an Allegra X-30R centrifuge (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Protein assay was performed using a BCA assay kit, according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA).

Electron and fluorescence microscopy of isolated extracellular vesicles (APO_{p200})

A freshly prepared APO_{p200} pellet isolated from a 9 DPA apoplastic wash solution was isolated as described above and diluted 100 times in phosphate buffered saline (pH 7). One microliter of the resuspended vesicles representing APO_{p200} was negatively stained with 1% uranyl acetate on plasma-treated copper grids. Imaging was performed using an accelerating voltage of 40 to 100kV on a Phillips CM-100 Transmission Electron Microscope (Philips/FEI Corporation, Eindhoven, Holland).

Glycome profiling

APO_T was fractionated into APO_{S200} and APO_{P200} fractions by ultracentrifugation as described previously [38]. The supernatant (APO_{S200} fraction) after ultracentrifugation was lyophilized completely. The pellet containing apoplastic vesicles (APO_{P200} fraction) were lysed by sonication and ultracentrifuged to collect the soluble fraction with the released polysaccharides [57]. The lyophilized APO_{S200} and APO_{P200} fractions were redissolved in water, the sugar contents were measured, and 2 µg of polysaccharides were spotted in each well of a 96-well plate and probed with 71 different antibodies as described previously [46]. The crude microsomes obtained from lysed fiber cells (P200) were processed identically and used as an additional control.

LC-MS/MS sample preparation

For LC-MSMS analysis, proteins were digested using trypsin as described previously [38,58]. We used all protein samples we gained from the APO_T, and S200 preparations, while 50 µg of proteins in the P200 cell fractions. Denatured protein samples were reduced in 10 mM DTT for 45 min at 60°C, and then alkylated with 20 mM iodoacetamide for 45 min at room temperature under the dark. The urea concentration of the solution was brought down to below 1.5 M by additional ammonium bicarbonate before trypsin (Sigma-Aldrich, St. Louis, MO, USA)) digestion. The digestion was proceeded at an enzyme-to-protein ratio of 1 to 50 at 37 °C. After overnight digestion, trifluoroacetic acid (TFA) was added to end digestion. The digested peptides were purified using C18 Micro Spin Columns 74-4601 (Harvard Apparatus, Holliston, MA, USA). One µg of each peptide sample was injected onto the LC-MS/MS system.

LC-MS/MS data acquisition

LC-MS/MS analysis was carried out as described previously [59]. Peptide samples were analyzed by a reverse-phase LC-ESI-MS/MS system using a Dionex UltiMate 3000 RSLCnano System coupled with the Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were loaded onto a trap column (300 µm ID × 5 mm) packed with 5 µm 100 Å PepMap C18 medium and then separated on a reverse phase column (75 µm ID × 50 cm) packed with 2 µm 100-Å PepMap C18 medium (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were resolved at a flow rate of 200 nL/min over a 160-min LC gradient. All the data were acquired in the positive ion mode in the Orbitrap mass analyzer using a higher energy collisional dissociation (HCD) fragmentation scheme. The MS scan range was from 375 to 1,500 m/z at a resolution of 120,000. The automatic gain control target was set as standard, maximum injection time (50 ms), dynamic exclusion 60 s, and intensity threshold

5.0e3. MS data were acquired in data-dependent mode with a cycle time of 3 s/scan. MS/MS data were collected at a resolution of 7,500.

Peptide Identification and Quantification

Andromeda search engine on MaxQuant (version 1.6.14.0) was used for relative protein abundance quantification and protein identification [60]. The search was conducted as described [61]. To minimize locus identity (Locus ID) assignment errors between cell fractions LC/MS raw files were searched in a single MaxQuant job, and signal intensities were normalized [60,62] across all time points (Supplementary Data 2). The three different parameter groups for the three cellular fractions were defined to specify different parameters for each cell fraction. The search parameters were as follows: the match between runs function was set with a maximum matching time window of 0.7 min as default; proteins identified by a single unique peptide were selected; Label-free quantification was selected within each parameter group; all other parameters were set as default. The cotton reference was built to facilitate transcriptome and proteome data integration [45]. To promote consistent locus ID assignments between annotated *Gossypium hirsutum* genome annotations, a locus ID conversion table is provided. Identified peptides, proteins, and a locus ID conversion table can be found in Supplementary Data 2.

Data filtering for protein identification in each cellular fraction (APO_T, P200, S200)

Two layers of filtering strategies were applied for each cellular fraction dataset to identify proteins in each cellular fraction as described before [38]. First, those proteins identified with MS/MS count(s) ≥ 1 in at least 2 out of 3 replicates were chosen as identified proteins in each cellular fraction. Secondly, quantified proteins with valid LFQ values in at least 2 out of 3 replicates in each cellular fraction were selected from the above count-based datasets. These reproducible proteins defined by MS/MS count(s) and LFQ criteria are provided in Supplementary Data 3a and 3b, respectively.

Profile interpolation

Up to 2 missing values, which are denoted as zeros, at the two ends or in the middle of a profile were interpolated. In the middle of a profile, a single zero was interpolated by averaging its neighboring non-zero values. In cases where two consecutive zeros occur within an abundance profile, a linear interpolation method was applied. At the ends of a profile, a single zero was replaced by its neighboring non-zero value, while two consecutive zeros were directly replaced by the nearest non-zero value. The interpolated profiles were used in downstream analyses and are available in Supplementary Data 3c.

Self-organizing maps (SOM) for protein expression patterns

Self-organizing maps (SOM), a robust unsupervised machine learning method [44], were used for analyzing protein expression patterns. We applied a SOM algorithm [43], based on an inner product distance metric, to cluster the interpolated protein profiles of each cell fraction. The SOM clusters defined the corresponding protein expression profile patterns (Fig. 2a). The SOM clustering results for the protein profiles in each group are available in Supplementary Data 4 and Extended Data Fig 2–4. Cluster profiles were flagged as unreliable if they reflected the overall distribution pattern of protein coverage (Supplementary Data 3d) or were dominated by patterns that can be explained by the interpolation, e.g., a gap of three consecutive zeros. After this inspection, 12 groups in APO_T, 23 groups in P200, and 9 groups in S200 fractions were selected as containing informative protein profiles (Supplementary Data 4).

Quality control for reliable protein expression patterns

For each SOM cluster, outliers and unreliable protein profiles were removed. Outliers were identified by calculating all pairwise similarities defined by the inner product of two profiles in a cluster:

$$\frac{x_i^T x_j}{\sqrt{x_i^T x_i}\sqrt{x_j^T x_j}}$$

If the similarity between a protein profile within a group and the group centroid was less than either the group's median similarity or 0.8, the protein was treated as an outlier and removed (Step 1, Fig. 2b). Additionally, unreliable profiles, defined as isolated non-zeros, were moved from the group (Step 2). This includes profiles with either a single non-zero value between two zeros or three consecutive zeros in the middle. Low abundance, unreliable profiles were identified based on signal distributions (Extended Data Fig. 5). When the averaged abundances across DPAs fall below 20 for APO_T, 19 for P200, and 20 for S200, these proteins tend to exhibit more zeros in their profiles. The cutoffs for low abundance proteins were determined using the median abundance values of proteins removed during earlier filtering steps (Step 3). reliable profiles are expected to have an averaged protein abundance of at least 20 (log2-transformed). In this dataset a 10 % increase or decrease in abundance in the log2 scale corresponds to a 4-fold change. To calculate average abundance profiles for protein groups, member intensities were first normalized to a 0–1 range (based on their own maximum value within the group) and then averaged, ensuring all profiles share the same scale. This set of 44 cell fraction protein expression group patterns (12 groups in APO_T, 23 groups in P200, and 9 groups in S200) is provided in Supplementary Data 5.

Merging protein expression patterns for data integration.

Similar protein expression patterns were merged into modules through additional SOM clustering analysis, using the 44 previously generated protein expression group patterns. We tested different SOM methods with 9, 20, 30, and 40 nodes to optimize node number in the SOM analysis (Supplementary Data 7a and Extended Data Figs. 6–9). As the number of nodes increased, the SOM produced more empty clusters or clusters containing only a single group pattern, typically with flat profile patterns. Therefore, we selected the 20-cluster SOM method, which defined 15 distinct clusters as merged protein expression modules (Fig. 3a). The profiles of these merged modules, along with the proteins in each module, were used as protein phenotypes in data integration analyses with phenotypic and transcriptomic data, which are available in Supplementary Data 7b,c.

Correlation analysis and classification of mRNA and protein pair

In this study, we used a published cotton fiber transcriptome dataset, which profiled transcripts present in developing fiber tissues obtained in the same time window [45]. We downloaded Supplementary Table 1 which includes transcript profiles of 74,776 genes and further filtered out low abundance transcripts with less than one transcript per kilobase million (TPM) to generate high-quality, reliable transcriptome information. Supplementary Data 11 provides the filtered 50,512 protein-coding genes used in this comparison analysis.

MaxQuant assigns proteins into a single protein group if they share all their peptides with other proteins [60], so ambiguously identified homoeologous or orthologous proteins within a protein group can limit to link proteins and their mRNAs. To address this, we used unique peptide information to assign representative proteins to each protein group. In this assignment, if proteins were detected by at least a quarter of the maximum number of unique peptides found in the leading protein of a protein group, those

proteins were considered identified for that group. Isoform identifications were then condensed to representative gene model IDs used in the transcriptome dataset.

Based on the homoeolog/ortholog assignment results, protein and mRNA pairs were classified into three cases for Pearson correlation coefficient (PCC) analyses. Case 1 includes homoeologs that were identified unambiguously, where unique peptides map to only one homoeolog. Case 2 involves cases in which unique peptides map to both homoeologs; in this case, we retained the homoeolog with the higher PCC. Case 3 includes homoeologs and orthologs identified by unique peptides that map to multiple homoeologs and more than one ortholog within a protein group. Using this protein assignment (annotated in Supplementary Data), we integrated the protein profiles (in protein expression groups shown in Fig. 2c) with their mRNA profiles and calculated PCCs between mRNA-protein pairs in each of the cell fractions (Extended Data Fig. 10–12 and Supplementary Data 12).

RNA-protein relationship patterns via SOM

To identify relationships between mRNAs and proteins that exhibit constitutively expressed profile patterns, P200 proteins in Case 1 with flat profiles were paired with their corresponding transcripts. We applied SOM again to cluster these pairs of protein and mRNA profiles. In this analysis, 100 nodes (10 rows and 10 columns) were used (Extended Data Fig. 13). The SOM clusters defined the relationship patterns between mRNAs and proteins over cotton development. Details of the clustering results and the profiles of paired mRNAs and proteins are provided in Supplementary Data 13.

Statistic tests and data analysis

Statistical analysis was performed using R version 4.2.0 [63] on RStudio 2022.07.1 [64]. The Flexible Self-Organizing Maps in Kohonen 3.0 package for R [43]. Microsoft Excel on Office 365 for Mac was used to organize and display the analyzed data.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [65] with the dataset identifier PXD051704.

Code availability

The source codes for the protein SOM clustering analyses and the weighted cross-correlation SOM clustering analysis are publicly available on GitHub (https://github.com/yangpengchengstat/Proteomics-based-models-of-cotton-fiber-protein-dynamics-and-gene-expression-control).

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Ethics declarations:

Competing interests

The authors declare no competing interests.

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Extended data

Extended Data Fig. 1. Enzyme coverages of metabolic pathways.

Extended Data Fig. 2. SOM grouping and profile filtering (APO_T).

Extended Data Fig. 3. SOM grouping and profile filtering (P200).

Extended Data Fig. 4. SOM grouping and profile filtering (S200).

Extended Data Fig. 5. Analysis of proteins removed at each data filtering step while identifying the subset of reliable members.

Extended Data Fig. 6. Merged SOM protein expression modules (9 cluster model)

Extended Data Fig. 7. Merged SOM protein expression modules (20 cluster model)

Extended Data Fig. 8. Merged SOM protein expression modules (30 cluster model)

Extended Data Fig. 9. Merged SOM protein expression modules (40 cluster model)

Extended Data Fig. 10. Profiles of paired mRNA and proteins (APO_T)

Extended Data Fig. 11. Profiles of paired mRNA and proteins (P200)

Extended Data Fig. 12. Profiles of paired mRNA and proteins (S200)

Extended Data Fig. 13. Weighted cross-correlation SOM of paired mRNA and proteins (P200)

Supplementary information

Supplementary Video 1. A movie of the cotton flower and boll development (-5 to 53 DPAs)

Supplementary Data 1. Fiber protein yields in the APO_T, P200, and S200 fractions.

- The protein amount yielded in each DPA from three different cellular fractions was measured by BCA assay.

Supplementary Data 2. Raw abundances of peptides and proteins (APO_T, P200, and S200).

- Raw peptide and protein abundance information obtained from a MaxQuant search were reported.

Supplementary Data 3. Raw and interpolated protein profiles across fiber development (APO_T, P200, and S200).

- The averaged abundances of proteins identified by MS/MS count(s) > 0 in at least 2 out of the 3 replicates and quantified by LFQ > 0 in at least 2 out of the 3 replicates
- **Interpolated protein profiles**
- Locus ID converter from Gorai to Gohir ID system
- Numbers of proteins at each filtering step, i.e. raw (unfiltered), MS/MS, and LFQ.

Supplementary Data 4. SOM clustering results and filtering for generating protein expression group profiles (APO_T, P200, and S200).
- SOM group profiles in each cell fraction
- **Profiles of members in each SOM group and data filtering were summarized.**

Supplementary Data 5. SOM protein expression group profiles (APO_T, P200, and S200).
- **The SOM groups with their profiles in the three cell fractions were reported and visualized in Fig. 2C.**

Supplementary Data 6. Gene ontology enrichment analysis of P200 and S200 protein expression groups with increasing patterns.

Supplementary Data 7. Merged SOM protein expression modules.
- Optimization of the number of groups defined for SOM-based clustering
- **Informative merged SOM groups with their profiles and profiles of members in each merged SOM group were reported.** The reliable profiles reported in Supplementary Data 4 were pooled, so localization and cell fraction group information can be found in column O.
- M14 APO_T analysis

Supplementary Data 8. Integration analyses of fiber phenotypes and merged protein expression modules.
- Phenotype estimation data (profiles of each phenotype from 6 to 24 DPA)
- PCC and p-values of correlation result between phenotypes and merged modules. This was visualized in Fig. 3c.

Supplementary Data 9. Gene ontology enrichment analysis of the M16 protein expression module.
- This was visualized in Fig. 3d.

Supplementary Data 10. Glycome profiling of polysaccharide epitopes (APO_{p200} vs. APO_{s200} vs. P200)
.
Supplementary Data 11. Abundance profiles of transcripts in cotton fiber across development [45].
- Filtered mRNA dataset downloaded from Grover et al, 2024 paper.

Supplementary Data 12. Analyses of Pearson correlation coefficient (PCC) between mRNAs and proteins present in cell fraction protein expression groups with trend-up or -down patterns.

Supplementary Data 13. Weighted cross-correlation SOM of paired mRNA and proteins (P200).

- SOM group profiles with fold changes in their expression profiles. This was used for data visualization in Fig. 4c.
- mRNA and protein profiles were assigned in each SOM group

Supplementary Data 14. Integrative analysis of mRNAs and proteins annotated as CW-related gene families.

- The list of Cell Wall Related Gene Families was downloaded at https://cellwall.genomics.purdue.edu/
- Data integration and correlation analysis results of CW-related gene families with their mRNA and protein profiles were reported.

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