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Label-Free Quantitative Proteomics Reveals Key Enzymes in Fiber Maturation

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Abstract Cotton fibers are important natural textile raw materials, and their maturation process directly affects the length, strength and quality of the fibers. However, the molecular regulatory mechanisms during the fiber maturation period remain unclear, which limits the genetic improvement of high-quality cotton varieties. To deeply reveal the protein regulatory network related to fiber maturation, in this study, Label-Free Quantitative Proteomics technology was adopted to systematically analyze the protein expression profiles in cotton fibers at different developmental stages. Further functional enrichment and protein-protein interaction network analysis indicated that cellulose synthase (CESA), sucrose synthase (SUS), peroxidase, heat shock protein, etc. play a core role in the process of fiber maturation. This study systematically analyzed the developmental biological basis of cotton fiber maturation, the types and expression characteristics of key enzymes, and verified the expression patterns of key genes through case studies. Finally, it explored the application potential of proteomics data in breeding. This research not only enriches the understanding of the maturation mechanism of cotton fibers, but also provides potential functional gene resources for molecular breeding of high-quality cotton.

Keywords Cotton fiber maturation; Label-free quantitative proteomics; Key enzyme; Cell wall synthesis; Molecular breeding

1 Introduction

Why is cotton important? It's not because it is widely grown, but because the fibers it produces support a multi-billion-dollar global market. The length, strength and fineness of fibers-these seemingly technical indicators-actually directly determine the quality of clothes and fabrics, as well as the commercial value of cotton. For industry and agriculture, cotton is both the protagonist and the foundation (Lee et al., 2024). From this perspective, to maintain the stability and competitiveness of the textile industry, merely having output is not enough; the quality of fibers must keep up.

But it's easy to say, but it's not easy to actually improve cotton fibers at the genetic level. Although we have gained a considerable understanding of the development mechanism of cotton fibers, when it comes to the "maturity" stage, the regulatory details at the molecular level remain unclear. For instance, how the structure of the cell wall changes and what specific roles proteins play have not yet been fully clarified. Traditional research methods have also made attempts, but they are relatively limited in terms of dynamic monitoring of protein changes. At this point, label-free quantitative proteomics emerged as a breakthrough. It does not rely on preset labels and can comprehensively and systematically analyze the abundance and function of proteins in fibers, and is less likely to miss important information (Distler et al., 2016; Ankney et al., 2018). Through it, key enzymes and regulatory proteins involved in fiber development are also more easily identified, which has greatly promoted our understanding of fiber maturation. Although this method seems to have a relatively high technical threshold, it can indeed provide many clues for the cultivation of superior cotton varieties in the future.

This study utilized a label-free quantitative proteomics process to analyze the proteome of cotton fibers at different developmental stages. By isolating fibroblasts and employing advanced mass spectrometry techniques, proteins crucial for fiber maturation were identified and located. This study aims to reveal the key enzymes and regulatory pathways that drive fiber development, providing a comprehensive framework for future crop improvement. These findings are of great significance to both basic plant biology and the agricultural industry, providing new strategies for improving fiber quality and yield.

2 Developmental Biology of Cotton Fiber Maturation

2.1 Developmental stages of cotton fiber and characteristics of the maturation phase

Not all types of fibers mature at the same time. The length of this process can vary significantly among different genotypes. Generally speaking, the development of cotton fibers can be roughly divided into several overlapping stages: initiation, elongation, secondary cell wall (SCW) synthesis, and final maturation. What truly affects the quality is this last step. At the mature stage, cells have basically completed cellulose deposition and are no longer living cells but transform into hollow structures. At this time, the dehydration process is also underway, which has a significant impact on the strength, dyeability and yield of the fibers. Fibers that are not fully mature have weak strength and uneven dyeing, which will limit their textile applications. Mature fibers, however, exhibit excellent fineness and structural integrity (Prasad et al., 2022; Iqbal et al., 2023; Jareczek et al., 2023).

2.2 Dynamic changes in cell wall composition during maturation

After the fibers stop stretching, the most obvious change is the rapid thickening of the cell wall. At this stage, the composition of the cell wall undergoes a drastic transformation, with cellulose almost becoming the sole protagonist, and its content can reach up to 98% at its peak. In fact, behind this is the synthetic transformation from primary wall to secondary wall, and the time point of this transformation varies among different types of cotton. Between 17 and 24 days after flowering, many studies have been able to observe this critical window through methods such as Fourier transform infrared spectroscopy (Abidi et al., 2010; Jareczek et al., 2023). The structure of the wall, such as its thickness and crystallinity, ultimately determines whether the fibers can meet the standards. Therefore, it is not the case that the more deposits there are, the better. It also depends on whether the organizational structure is reasonable.

2.3 Metabolic shifts and regulatory signals in the maturation process

During the period of fiber maturation, metabolism within cells is not static. On the contrary, at this time, metabolic pathways actually undergo a "role transformation". At the beginning, energy was mainly used in aspects such as cell expansion and relaxation of wall structure. But once it enters the mature stage, the system will shift its focus to the synthesis of cellulose and the thickening of cell walls. This transformation is controlled by multiple signals, among which several plant hormones play a leading role, such as abalic acid, auxin, gibberellin, brassinolide, etc. Transcriptomic and proteomic studies have identified many gene modules and expression patterns related to these hormonal pathways (Gou et al., 2007; Xiao et al., 2019). Of course, hormones alone are not enough. What truly determines the final outcome is the coordination of multiple levels of regulation, including transcription factors, enzyme activity, and signal integration. If well regulated, the output and quality of fibers will be more stable (Jan et al., 2022; Grover et al., 2025).

3 Categories and Functions of Key Enzymes Related to Fiber Maturation

3.1 Cell wall biosynthetic enzymes

Ultimately, the "building" process of cell walls plays a significant role in whether cotton fibers can grow strong or not. Enzymes like cellulose synthase exert the greatest force during the secondary wall formation stage, directly determining the strength and structure of the fiber. Rather than all the enzymes involved in the process being active at the same time period, such as β-1, 3-glucanase (GhGLU18), which mainly exerts its force in the late elongation stage and during secondary wall synthesis. Once the expression level of this enzyme increases, the accumulation of polysaccharides and the thickening of the cell wall will also be significantly enhanced. Conversely, if it is inhibited, a phenotype of short fibers and weak structure will be observed. GhGLU18 is also regulated by the NAC transcription factor GhFSN1. This hierarchical relationship indicates that it is not a supporting role in cell wall metabolism (Fang et al., 2024). As for cinnamyl alcohol dehydrogenase (GhCAD37A/D), its role is to participate in the synthesis of lignin monomers. When the lignin content changes, the mechanical properties of the fiber will naturally change accordingly (Li et al., 2024).

3.2 Oxidoreductases and energy metabolism enzymes: regulation of respiratory chain and antioxidant systems

Oxidative stress has always been there, but not all enzymes can handle it. On the path of fiber maturation, ascorbic acid peroxidase (APX) becomes particularly important. Different APX subtypes come into play at different stages.

For instance, GhAPX10A, GhAPX10D, GhAPX12A and GhAPX12D become active only at the mature stage. They mainly regulate H_2O_2 levels to prevent oxidative damage from disrupting cell wall synthesis (Zhu and Luo, 2024). Experimentally, when APX activity is high, the levels of H_2O_2 are usually relatively low, which indicates that these enzymes do indeed play a protective role. In addition, energy metabolism enzymes such as ATP-dependent phosphofructokinase and glucose-6-phosphate dehydrogenase are also indispensable. They ensure that metabolism does not break down during the high-energy demand stage of fiber construction.

3.3 Hormonal and signal transduction-related enzymes: enzymes involved in ABA and JA pathways

It is no longer news that fiber maturation is regulated by hormones, but the enzymes involved are far more complex than we imagine. Take ethylene as an example. There is an E3 ubiquitin ligase (GhXB38D) that affects the entire pathway by regulating the stability of ethylene synthases (GhACS4, GhACO1). The result is that when GhXB38D is inhibited, ethylene is synthesized and the fibers grow better (Figure 1) (Song et al., 2023). Another key player, GhLCBK1, which is related to auxin synthesis, has also been proven to affect fiber elongation. In addition to ethylene and auxin, "established" signaling hormones such as ABA and JA were also present. Some related enzymes were involved, helping plants adjust their maturation rhythms in the face of environmental fluctuations (Tao et al., 2018; Zhang et al., 2024).

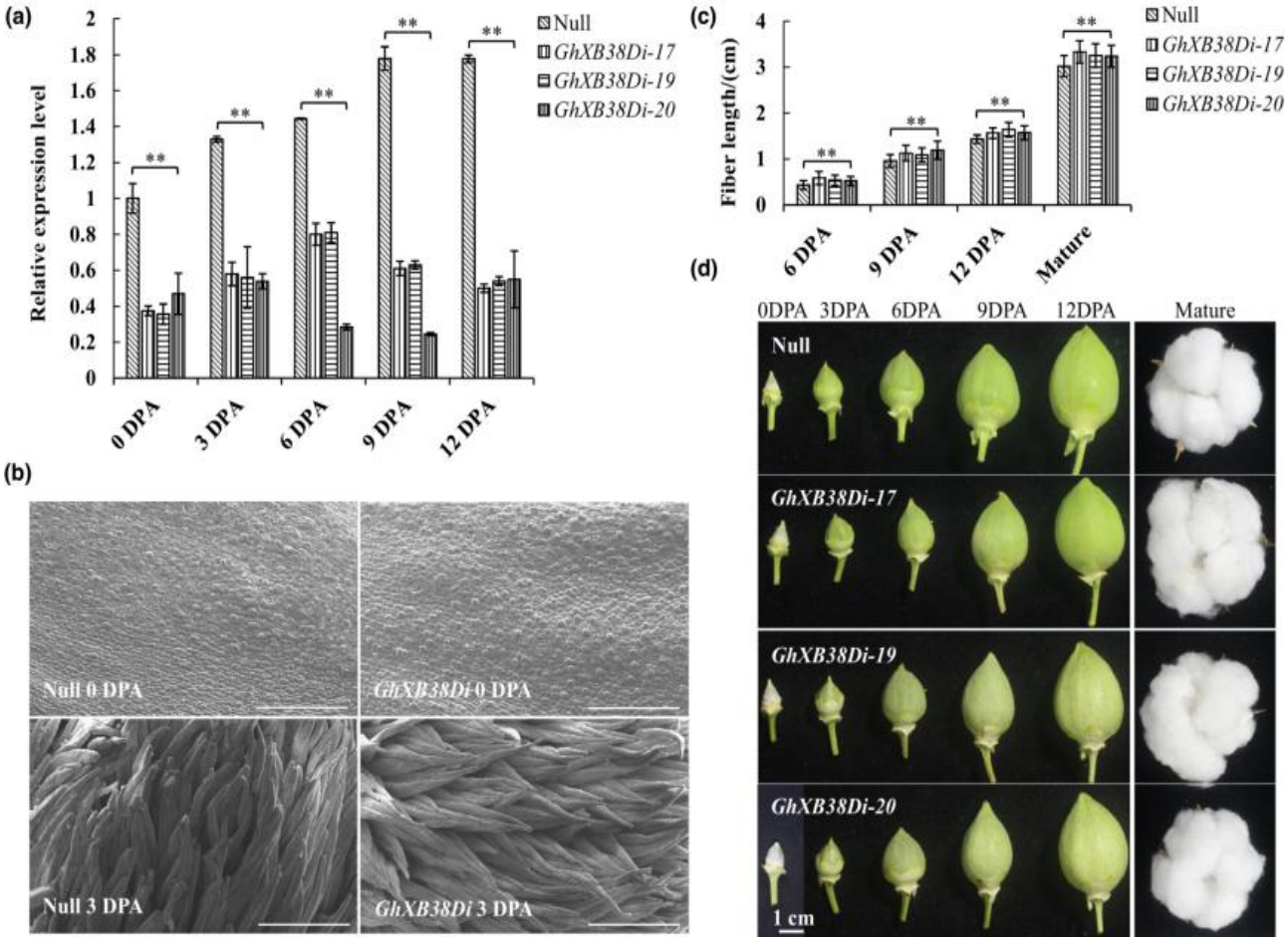

Figure 1 Suppression of *GhXB38D* expression in cotton promotes fibre elongation (Adopted from Song et al., 2023)

Image caption: (a) Relative expression level of *GhXB38D* in null plants and *GhXB38D* RNAi lines (*GhXB38Di-17*, *19* and *20*) during fibre development (from 0 to 12 DPA). Error bars represent ± SE of three biological replicates (*$P < 0.05$; **$P < 0.01$, by Student's *t*-test). (b) Scanning electron microscope (SEM) images show the ovule surfaces of null plants and *GhXB38Di* lines at 0 and 3 DPA. SEM images show the epidermal cells in the centre of the ovule. Bars = 100 μm. (c) Average fibre length of null plants and *GhXB38D* RNAi lines (*GhXB38Di-17*, *19* and *20*). More than 100 seeds per line were used for statistical analysis. Error bars represent ±SE of three biological replicates (*$P < 0.05$; **$P < 0.01$, by Student's *t*-test). (d) Comparison of cotton bolls from null plants and *GhXB38Di* lines (*GhXB38Di-17*, *19* and *20*) during fibre development (from 0 DPA to 12 DPA and at maturity). Bars = 1 cm (Adopted from Song et al., 2023)

4 Expression Patterns and Regulatory Mechanisms of Key Enzymes

4.1 Spatiotemporal expression patterns of key enzymes during fiber maturation

Not all enzymes are "online 24/7" during the maturation process of cotton fibers. The expression of different isomers of metabolic enzymes such as cytoplasmic pyruvate kinase often depends on specific tissues and specific developmental stages; in other words, they are on demand. They are not only controlled at the transcriptional level but also constrained by post-transcriptional mechanisms. The entire regulatory process is very detailed, with the aim of keeping up with the specific requirements of fibroblasts for energy and carbon flow at different maturation stages (Wulfert et al., 2020; Wang and Zhang, 2024). Under this dynamic mechanism, enzyme activity is actually constantly being "regulated" and "adjusted".

4.2 Effects of post-translational modifications on enzyme activities

However, sometimes even if genes are expressed, enzymes may not take effect immediately. This involves the "switch" of the post-translation modification (PTM) layer. Modifications such as β -hydroxybutyrylation, acetylation or methylation of lysine can significantly alter the activity or stability of enzymes. For example, acyltransferase p300 can add a β -hydroxybutyryl group, and HDAC1/2 is responsible for removing them-these alterations may seem minor, but they are key actions in regulation, especially in scenarios of rapid switching of metabolic flux or stress response (Hemmerlin, 2013; Huang et al., 2021; Wang et al., 2022). Such modifications do not only act on the enzymes themselves, but may also be involved in a host of pathways such as DNA repair and metabolic regulation.

4.3 Comparative analysis of key enzyme expression among different cotton varieties

In fact, even if they are all cotton, different varieties have quite different expressions of key enzymes. This is not merely a difference at the transcriptional level, but also involves the expression intensity and modification mode at the proteomic level. Studies have found that there are significant differences in the expression of some metabolic enzymes and regulatory enzymes among different cotton species, which directly affects the maturation rate and quality of fibers (Zanger and Schwab, 2013; Zelezniak et al., 2018). This kind of difference often stems from the underlying genetic variations and variations in regulatory networks. It is precisely for this reason that the combination of quantitative proteome data with genotype and phenotype information can more comprehensively depict the functional performance of different varieties and provide real and applicable references for breeding.

5 Case Studies: Functional Validation and Application of Candidate Genes

5.1 Expression verification of representative enzymes (e.g., GhCESA4, GhSUS) in cotton fibers

To determine whether a gene is "truly useful", merely looking at its sequence is not enough; it is necessary to see if it has "come online" at the critical period. Genes such as *GhCESA4* (cellulase synthase) and GhSUS (sucrose synthase), which are suspected to be involved in fiber maturation, are often confirmed by researchers for their expression through quantitative RT-PCR or transcriptome analysis. As long as they are active in a specific tissue or developmental stage, there is basically a reason to dig deeper (Figure 2) (Huang et al., 2021).

5.2 Gene knockdown (VIGS) and transgenic analyses reveal regulatory roles in fiber maturation

A high level of expression doesn't necessarily mean functionality; one still needs to "give it a try". So, researchers often use some tools, such as VIGS (virus-induced gene silencing) or RNA interference, to first "turn off" a certain gene and see what the reaction is. Some choose to overexpress it or directly knock it out to observe whether it will affect the fiber length, strength or cell wall composition. If a certain trait changes, it is highly likely that this gene does have a regulatory role (Alinezhad et al., 2016; Rohde et al., 2018; Cornean et al., 2021). Nowadays, there are still many high-throughput in vivo systems and model organisms available. It is not a problem to screen a large number of genes at one time, and the efficiency is much faster than before (Zhu et al., 2017; Cornean et al., 2021).

5.3 Trait association studies and QTL integration for fiber quality improvement

But verification is not enough. If these genes do not "play a role" in actual breeding materials, then they have no promotion value either. Therefore, many studies will combine functional verification with QTL analysis or GWAS. In this way, not only can it be confirmed that the gene is "properly expressed", but also whether it appears together with the ideal trait, which is known as "co-isolation" (Albert and Sauvage, 2022). If the genes screened out can be

overexpressed and associated with phenotypes simultaneously, they will be of great value to be retained. The combination of these methods constitutes the truly "hardcore" tool for improving the fiber properties of cotton.

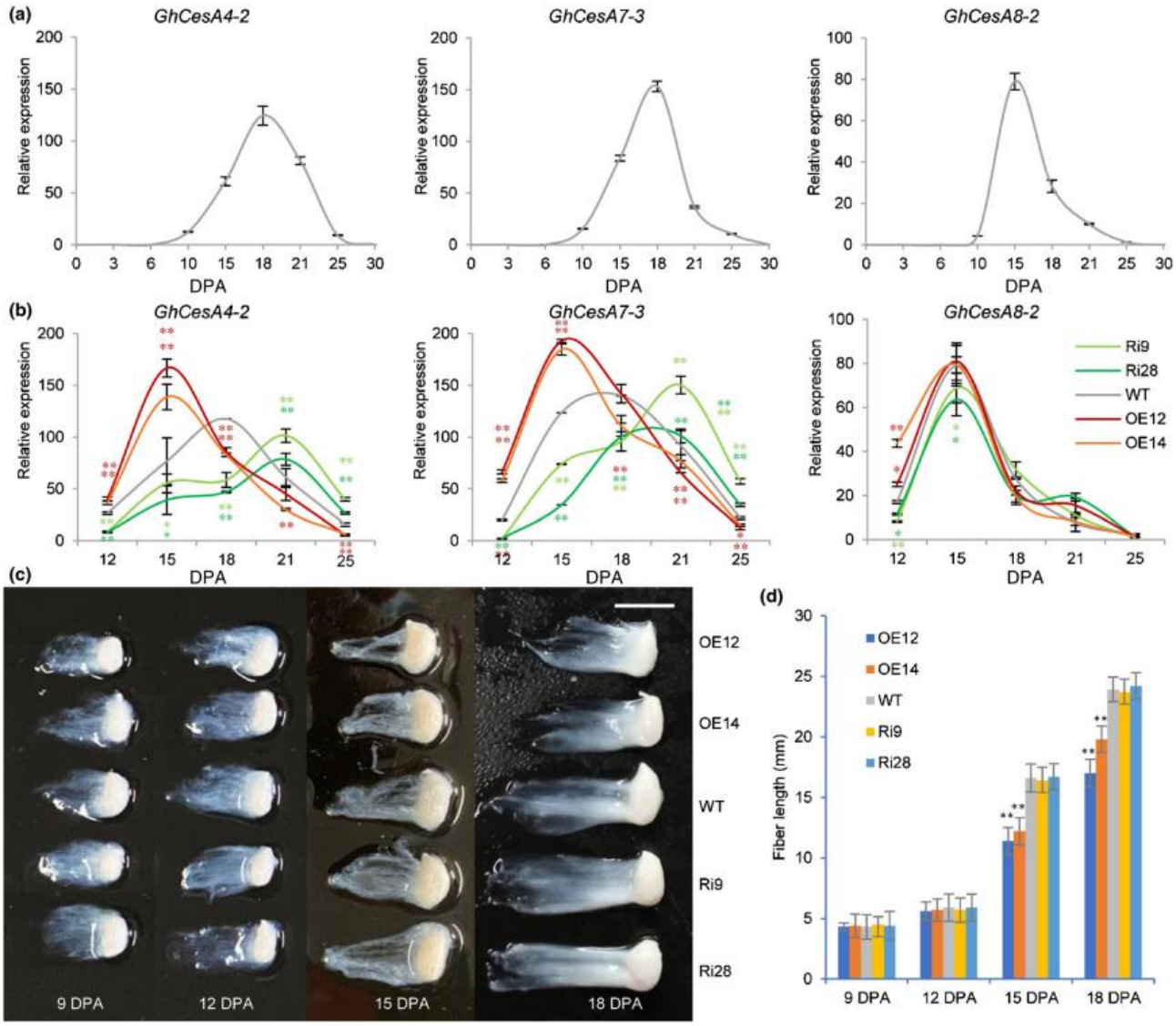

Figure 2 Changes in *GhMYB7* expression affect expression of *GhCesAs* and fibre elongation. (a) Expression of *GhCesA4-2*, *GhCesA7-3* and *GhCesA8-2* in wild-type cotton fibres, using *GhUBI1* as the internal control for normalisation. (b) Expression of *GhCesA4-2*, *GhCesA7-3* and *GhCesA8-2* in 12, 15, 18, 21 and 25 DPA fibres of transgenic lines and wild-type. (c) Image of fibres from wild-type and *GhMYB7* transgenic lines. Bar, 10 mm. (d) Comparison of fibre length between the wild-type and *GhMYB7* transgenic lines. Six cotton bolls located at the similar position in the plant were taken from each genotype, 10 ovules were separated from each boll to measure the fibre length. Values are means ± SD with three biological replicates. *, $P < 0.05$; **, $P < 0.01$ (Adopted from Huang et al., 2021)

6 Application Potential of Proteomics Data in Breeding

6.1 Prospects for developing protein biomarkers based on key enzymes

Some proteins are inherently linked to their properties, especially those key enzymes that play a role in the process of fiber maturation. Through proteomics, researchers can now identify and quantify the expression levels of such proteins more quickly, and this also provides a new alternative path for breeding projects. Indicators such as fiber strength or quality, which used to rely on phenotypic data, may now be able to "show signs" at the protein level in advance. Non-invasive and high-throughput, this type of detection method is also more flexible to use. In fact, similar methods have long been tried in animal breeding, such as for the screening of meat quality and reproductive traits (El-Hack et al., 2018; Gouletsou et al., 2022), with good results. This also indicates that this approach does not fit in when applied to cotton or other crops.

6.2 Integration of proteomic, phenotypic, and genotypic data for genetic improvement

Sometimes, merely looking at DNA sequences is not intuitive enough, especially when it comes to proteins that function only after translation. Proteomics fills this "blind spot", as it can help us discover modifications or abundance changes that have not been detected at the RNA level. If the protein data, phenotypic expression and genotype information are analyzed together, many previously unclear trait mechanisms will become much clearer. In this way, breeders can not only know where a certain trait "grows", but also understand the "regulatory logic" behind it, and then precisely select species (Das et al., 2015; Adnane et al., 2024). This kind of multi-omics combination approach has been attempted in the fields of animals and plants in recent years, and the feedback at the practical level has become increasingly positive.

6.3 Conceptual design of proteome-assisted selection (PAM) for precision breeding

The concept of proteomic assisted selection (PAM) has not been around for long, but it fills an important "gap" in traditional methods. In the past, we relied more on genotype and phenotype for seed selection. Now, if we can directly find clues from the proteome spectrum, it might be faster and more accurate. The core of PAM is to identify individuals with expression patterns that are strongly related to the target trait at the population level. It does not replace the existing methods but serves as a supplement-especially when phenotypic differences are linked to changes in protein function, this strategy is more advantageous (Das et al., 2015; El-Hack et al., 2018; Adnane et al., 2024). As the integration of proteomics data and other omics deepens, this approach may play a more direct role in ensuring food security and promoting breeding efficiency.

7 Conclusions and Outlook

It is difficult to figure out how cotton fibers mature just by omics alone. In recent years, the research on quantitative proteomics, genomics and transcriptomics has made a big step forward-genes like ACLA-1, VTC2 and GA2OX1 were screened out in such multi-dimensional data. These are all highly expressed during the critical period of fiber development, and they seem to be related to early maturity and fiber quality. The problem is that the genetic relationships among these traits are not always on the same side; sometimes they even interfere with each other. So, even though there are already some major QTLS and candidate genes, it is still not very easy for breeding strategies to balance yield, early maturity and quality. However, multi-omics integration has at least provided us with some clues behind fiber maturation, such as how dynamic processes like cell wall biosynthesis, hormone signaling, and cytoskeletal regulation are intertwined.

However, to be fair, proteomics is not that easy to handle in plants. Especially at the mature stage of cotton fibers, where the cell walls are thick and crystalline, extracting proteins is like picking water out of a stone. Not only is the yield low, but it is also very easy to lose those key regulatory proteins. Moreover, cotton itself is not a species with a simple genome. Polyploids and highly similar gene families all make the recognition and quantification of proteins complex. What's more troublesome is that once in the fields, the variations between different environments and the interaction between genotypes and the environment also make the interpretation of proteomic data less stable.

Looking ahead, it's not over yet. If proteomics is truly to play a greater role in the genetic improvement of cotton, it must be carried out in conjunction with genomics, transcriptomics, and phenomics. Multi-omics integration is a major trend. Only by piecing together these data can we more accurately identify those biomarkers and regulatory networks that are truly controlling fiber maturation and quality. New tools such as single-cell omics and high-throughput phenotyping techniques may also offer finer resolutions in the future. Combined with gene editing, perhaps we can breed a batch of new cotton varieties that not only have good fibers but also are resistant to environmental fluctuations.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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