



# iTRAQ-based quantitative proteomic analysis of cotton (*Gossypium hirsutum* L.) leaves reveals pathways associated throughout the aging process

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## Abstract

Leaf senescence is associated with changes in proteomics. In this study, to quantitatively analyze changes in protein abundance related to leaf senescence in cotton (*Gossypium hirsutum* L.), we employed a proteomic approach utilizing iTRAQ and physiological assays throughout the senescence of leaf tissue in cotton grown under typical field conditions. Physiological tests showed leaf chlorophyll content and photosynthetic rates decreased significantly throughout the aging process. A total of 195 differentially abundant proteins (DAPs) throughout leaf senescence were identified by mass spectrometry. Of these, 91 (47%) proteins were upregulated, while the remaining 104 (53%) were downregulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses indicated that the expression of genes in several pathways potentially associated with aging changed throughout senescence, including metabolic pathways, photosynthesis, pyruvate metabolism, nitrogen metabolism, and diterpenoid biosynthesis. Our findings provide a deeper understanding of aging in plants as well as fundamental data describing leaf senescence in cotton.

**Keywords** Cotton · iTRAQ · Leaf senescence · Proteome · Aging

## Introduction

Cotton (*Gossypium hirsutum* L.) is the world's remarkable oilseed source and is also a primary natural fiber source (Guo et al. 2017). Leaves are the major photosynthetic organ of cotton plants, and they are essential to plant growth and biomass accumulation, fundamental covariates of agricultural yields. Leaf senescence is a process that increases

plant productivity and is caused by a wide range of signal crosstalk that harmonizes environmental factors with the age-related internal mechanisms of plants (Allu et al. 2014; Quirino et al. 2000). However, premature senescence of leaves is a serious cause of crop yield reduction (Balazadeh et al. 2010). This phenomenon shortens the period of photosynthesis of plants and reduces the accumulation of photosynthetic products, resulting in reduced yield and quality (Liu et al. 2016a). Premature senescence can decrease fiber quality and fiber yields, with substantial negative impacts (Dong et al. 2006).

Some senescent plants grown under optimum conditions have higher chlorophyll content prior to their senescence and thus have prolonged lifespans. Chlorophyll degrading enzyme activity increases during leaf senescence, and photosynthetic pigments (i.e., carotenoids, lutein, and chlorophyll) fall faster; at the same time, some genes with integral roles in photosynthesis are downregulated, leading to a decrease in leaf photosynthetic capacity (Wu et al. 2012). These changes cause the leaves to turn yellow, which is the morphological sign of aging (Lim et al. 2007).

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The initiation and development of leaf senescence are accompanied by a large number of changes in gene expression, and the activation of a series of new genes is necessary for the onset of aging (Gepstein 2004; Zhang et al. 2010). In plants, hundreds of aging-related genes have been identified (Bhalerao et al. 2003; Andersson et al. 2004; Gepstein 2004; van der Graaff et al. 2006; Zhang et al. 2010). In addition, transcriptase analyses have revealed the complexity of regulation of leaf senescence (He et al. 2001). Multiple studies have also identified senescence-related genes by assaying transcriptomes, transcription factors and plant hormones in senescing leaves (Kong et al. 2013; He et al. 2001). However, few studies have focused on aging leaf proteomics.

Proteomes differ distinctly among tissues and cells, and the expression and modification of proteins are also affected by environmental factors and developmental stages. In recent years, with the development of ultra-high-performance liquid chromatography (UPLC) and mass spectrometry, the advantages of the isobaric tags for relative and absolute quantitation (iTRAQ) approach have become quite obvious. This approach has become a vital research technology for studying the molecular mechanisms of plant leaf protein responses, and iTRAQ can be used in high-throughput comparative proteomics analyses conducted across different development stages (Qin et al. 2016).

In this study, we investigated proteomic changes throughout the senescence of photosynthetically active leaves on the main stems of cotton plants grown under typical field conditions. The iTRAQ method was used to assess proteomic changes in leaves and to identify differentially expressed proteins, throughout the aging process. The bioinformatics method is used to analyze the differentially expressed proteins (DAPs) and reveal their biological functions. Further characterization of these DAPs will clarify the signaling pathways activated by cotton in response to and as a part of senescence.

## Materials and methods

### Plant materials

The field experiments were conducted in Baoding (38°49'N, 115°44'E), Hebei, China, during the 2016 growing season. The experimental area is located within the warm-temperate arid zone with a continental climate. 'Lumianyan 28' (*Gossypium hirsutum* L.), a commercial cotton cultivar that is widely cultivated in the Yellow River Basin of China, was used exclusively in this study.

Seeds were sown on April 25, 2016, and plants were harvested in late September 2016. Samples for tests were given priority to assay leaf stem function (using samples from the third main-stem leaf from the terminal bud). Samples were

collected on August 6, August 14, and September 1, at leaf ages of 18 days, 26 days, and 44 days, which are hereafter referred to as M1, M2, and M3, respectively. Immediately after collection, all tissue samples were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Pigment determination

The third main-stem leaves from the terminal bud in field-grown plants were assayed for pigment concentration. Chlorophyll was extracted from 0.1 g fresh leaf samples using 95% acetone and measured at 645 and 663 nm with a UV spectrophotometer (Shimadzu UV-2450 Kyoto, Japan) (Lichtenthaler 1987).

### Leaf gas-exchange measurement

The third main-stem leaves were subjected to gas-exchange analysis with the LI-6400XT Portable Photosynthesis System (Li-Cor Inc. Lincoln, NE, USA) to determine net photosynthesis. During the measurements, the determination conditions were as previously described by Pu et al. (2015).

### Protein extraction

To extract proteins, the prepared leaf samples were first ground into dry powder in a mortar with liquid nitrogen. Then, the dry powder was dissolved in 200  $\mu\text{L}$  of triethylammonium bicarbonate (TEAB) lysis buffer (pH 8.0, 8 M urea/100 mM TEAB) and ultrasonicated for 15 min. Then, centrifuge at  $10,000\times g$  for 20 min, submerged and incubated the supernatant for 2 h by the addition of four volumes of cold acetone containing 10 mM dithiothreitol (DTT). The mixture was then centrifuged at 12,000 rpm and  $4^{\circ}\text{C}$  for 20 min, the supernatant was discarded, and it was mixed with 800  $\mu\text{L}$  of acetone at  $56^{\circ}\text{C}$  to degrade the disulfide bond in the extracted protein. Next, the mixture was centrifuged again by the above centrifugation method and dried under vacuum. The collected dry precipitate was dissolved in 100  $\mu\text{L}$  of TEAB lysis buffer (Liao and Huang 2011), and then, the total protein concentration was measured using the classical Bradford method (Bradford 1976).

The stored protein sample was reduced with 10 mM DTT for 30 min at  $56^{\circ}\text{C}$ , then alkylated with 50 mM iodoacetamide in the dark for 30 min at  $50^{\circ}\text{C}$ , diluted fivefold, and finally, trypsin digested with 100 mM TEAB (Zhang et al. 2018).

### Digestion and desalting

One hundred micrograms of each protein sample were dissolved in lysis buffer and diluted with 50 mM  $\text{NH}_4\text{HCO}_3$  (500  $\mu\text{L}$ ) to reduce and alkylate the sample, followed by the

addition of 2  $\mu\text{g}$  trypsin, and incubated at 37 °C overnight to complete protein digestion. Next, an equal volume of 0.1% formic acid (FA) was added to the above solution to complete the acidification. Peptide samples were purified using a Strata-X C18 column (Phenomenex Inc., Torrance, CA, USA), first activated with methanol, equilibrated three times with 0.1 mL FA, then twice with 0.1% FA + 5% acetonitrile (ACN), and then eluted with 1 mL of 0.1% FA + 80% CAN. The eluted peptide was dried with a vacuum concentrator and then redissolved into 20  $\mu\text{L}$  of 0.5 M TEAB buffer solution for peptide labeling.

### Labeling and fractionation

The iTRAQ Reagent-8 plex Multiplex kit (AB SCIEX UK Limited, Warrington, UK) was employed to label the prepared samples. Reagents 113, 114, and 115 were used to label the M1 samples, and 118, 119, and 121 were used for the M3 samples. Next, the labeled samples were fractionated by HPLC (Thermo DIONEX Ultimate 3000 BioRS, Thermo Fisher Scientific, Waltham, MA, USA) using a Durashell C18 column (5  $\mu\text{m}$ , 100  $\text{\AA}$ , 4.6  $\times$  250 mm) to obtain 12 fractions.

### LC-MS/MS analysis

We performed LC-ESI-MS/MS data analysis with the AB SCIEX nano-LC-MS/MS system (Triple TOF 5600 plus, AB, Foster City, CA, USA). In this study, the method described by Yang et al. (2018) was applied for sample operation and condition settings. The quadrupole isolation window for the TripleTOF 5600 + mass spectrometer was 0.6–0.8 Da.

### iTRAQ protein identification, quantification, and bioinformatic analysis

ProteinPilot software v4.5 (SCIEX, Framingham, MA, USA) was used to analyze raw MS/MS file data. In protein identification, we integrated Paragon algorithm 2 into the ProteinPilot, was employed in queries against the NCBI *Gossypium* database (consisting of 182,674 items, as of January 2016). The parameters used for analyses were those that were previously described by Pu et al. (2015). For functional annotation, all DAPs were searched against the Gene Ontology (GO) (Conesa et al. 2005), as well as the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa and Goto 2000).

### qPCR analysis

The third main-stem leaf samples subjected to iTRAQ analysis were also used for RNA preparation. Total RNA

quantitative RT-PCR (qPCR) was performed as previously described (Chu et al. 2012). The qPCR was performed on an ABI7500 real-time system. All PCR primers were designed using Primer Express 3.0 and are shown in Table S1. Relative expression-level evaluation was conducted using the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen 2001).

## Results

### Pigment content and photosynthesis assays

Chlorophyll content is an important index of leaf senescence (Zhang et al. 2010). We investigated the chlorophyll content of the third main-stem leaves from the terminal apex at leaves aged 18 days, 26 days, and 44 days (Fig. 1) to characterize progress in leaf senescence. Leaf chlorophyll content considerably decreased with increasing leaf age. The total Chl contents at 26 days and 44 days were reduced by approximately 10.27% and 37.52%, respectively ( $p < 0.05$ ).

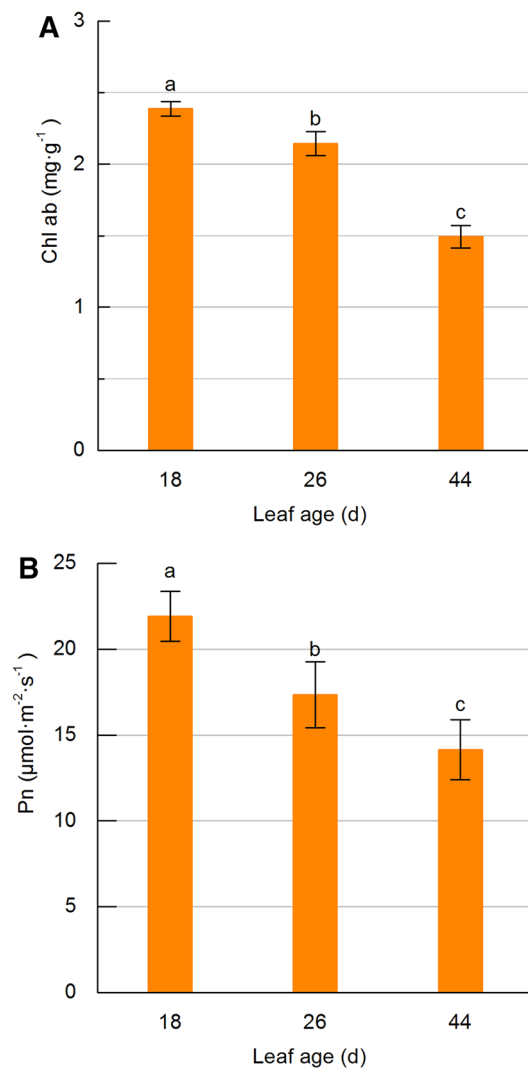
The trend in photosynthetic rate was consistent with that of chlorophyll content, decreasing with leaf age. The photosynthetic rates at 26 days and 44 days were reduced by approximately 20.84% and 35.47%, respectively ( $p < 0.05$ ).

The senescence of the leaves collected in 18 days, 26 days, and 44 days, corresponding to full maturity, medium-term senescence, and late senescence was determined according to changes in photosynthetic rate and chlorophyll content.

### Quantitative identification of cotton leaf proteins using iTRAQ

To further understand the molecular mechanism of cotton leaf senescence in field plots, the proteomic changes in third main-stem leaves were separately assessed for leaves aged at 18 days, 26 days, and 44 days. In addition, the number of proteins as well as their functional properties and associated pathways were analyzed.

The total spectra were comprised of 323,662 elements, based on three replicates. From this, 16,463 of the identified spectra matched known peptides. Ultimately, 16,463 peptides and 3882 proteins were identified, with 2705 proteins (69.68%) containing at least two unique peptides (Fig. 2a). A total of 1177 proteins matched one identified peptide, 918 matched 2, 148 matched more than 10, and the remainder matched 3–9 (Fig. 2a). Using the method of percentage of matched amino acids from the identified peptides, the protein sequence coverage of the particular protein was obtained (Fig. 2b), yielding an average coverage of 15.63%.



**Fig. 1** Photosynthetic performance of third main-stem leaves collected from cotton plants grown in field plots at three different developmental stages. **a** Total chlorophyll content. Data are means of nine biological replicates  $\pm$  SE. **b** Photosynthetic rate. Data are means of six biological replicates  $\pm$  SE. Different letters at the top of the bars in each plot indicate significant differences ( $p < 0.05$ ) between treatments

### Protein differences throughout leaf development

In the present research, any proteins with more than a 1.2-fold difference that was statistically significant were designated as significant DAPs. Accordingly, the number of DAPs was 195 (Table S2); among these, 91 (47%) were upregulated, while 104 (53%) were downregulated.

To understand the functional categories of DAPs, we performed a GO enrichment analysis on them. Classify DAPs based on biological processes, molecular functions, and cellular components (Fig. 3, Table S3), which included 23 biological process, 8 cellular component, and 10 molecular

function categories. The main biological functional categories represented were generation of precursor metabolites and energy, ribosome biogenesis, carbohydrate catabolic process, photosynthesis, and the photosynthetic electron transport chain. The cellular component categories represented were plastid, chloroplast, organelle, thylakoid, cytoplasm, plastid stroma, and photosynthetic membrane. DAPs were classified into binding, phosphoenolpyruvate carboxylase activity, and lytic enzyme activity, depending on their molecular functional properties.

Signal pathway analyses can help to clarify the biological functions of DAPs to determine the main biochemical pathways and signal transduction pathways through which proteins respond to age. Accordingly, these DAPs were further analyzed by the KEGG database and found to be mainly enriched in metabolic pathways (53.4%), photosynthesis (8.5%), pyruvate metabolism (6.8%), nitrogen metabolism (4.2%), diterpenoid biosynthesis (3.4%), carotenoid biosynthesis (3.4%), and galactose metabolism (3.4%; Table 1).

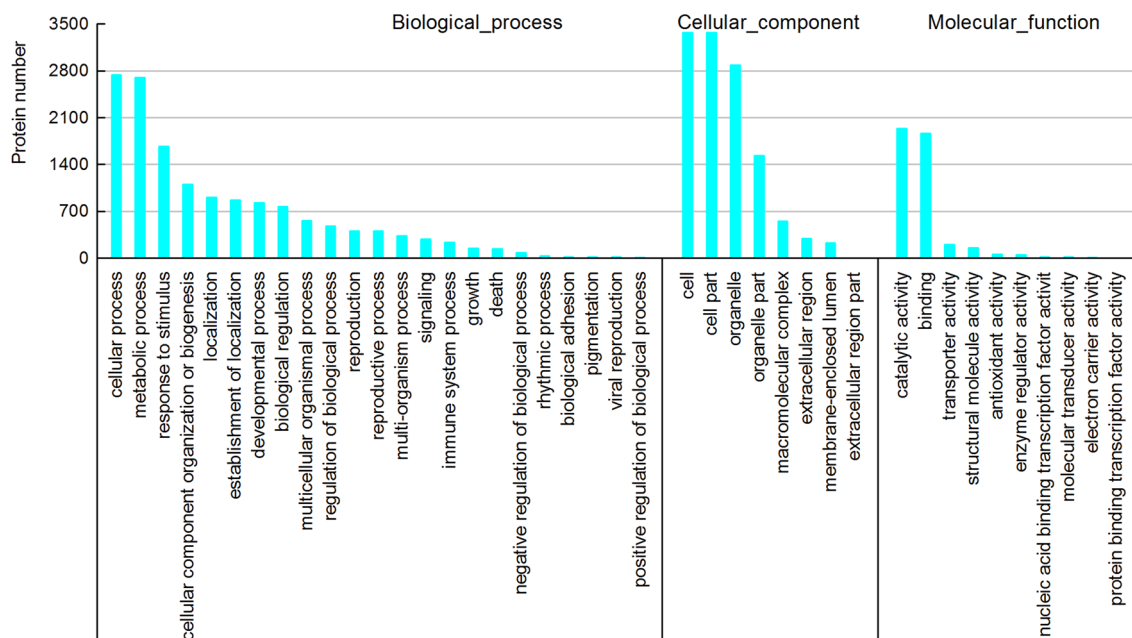
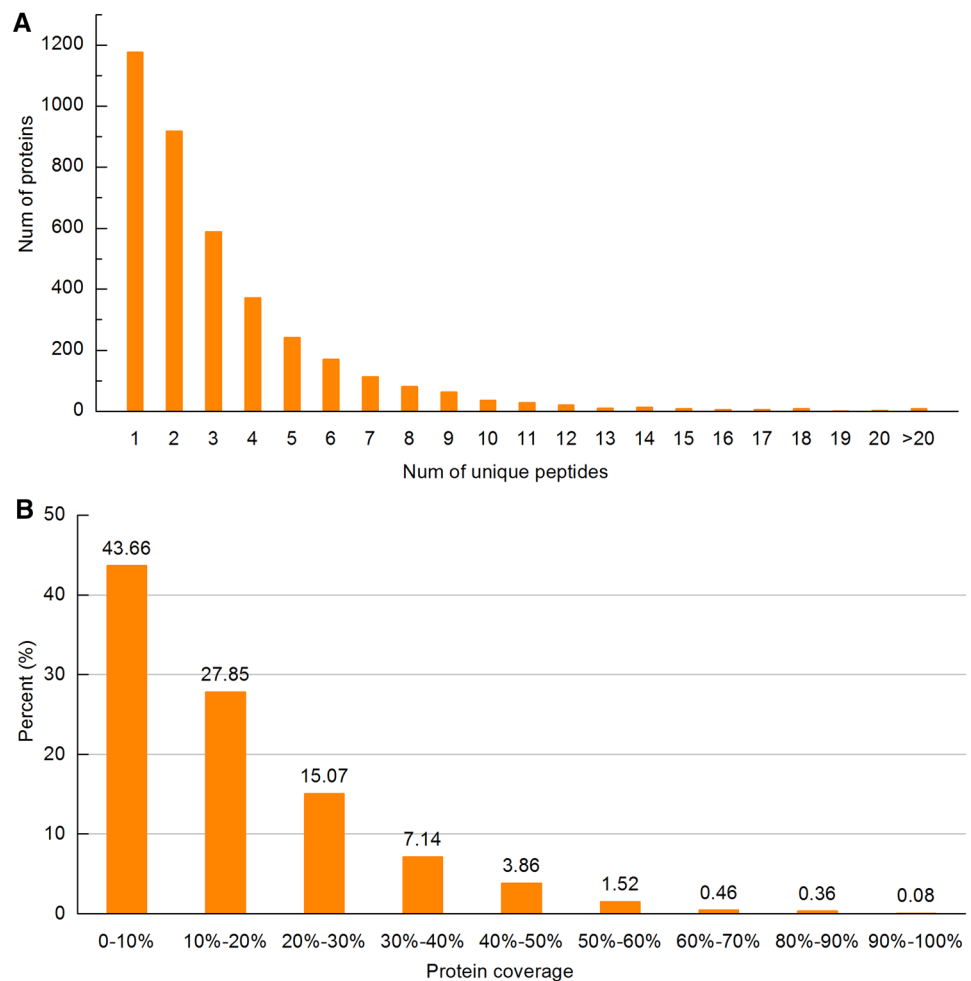
### Complementation of iTRAQ data with qPCR of selected candidates

From among all 195 DAPs, 10 were selected for transcriptional analysis (Fig. 4). Among these proteins, a majority showed consistent mRNA and protein expression patterns. However, two proteins exhibited contrasting expression patterns. This result may be due to differences in post-transcriptional, translational, or post-translational regulation. While most of the changes agreed with respect to sign, our results confirmed that mRNA transcript abundance does not correlate perfectly with protein abundance (Liu et al. 2016b). The results indicate generally parallel but flexible associations between mRNA and protein expression profiles in aging-associated proteins.

### Discussion

Proteomic methods for global expression analyses and protein identification in plants have become highly efficient in recent years (Qin et al. 2016). Thus, iTRAQ technology and LC-MS/MS have been employed together widely, demonstrating their proven value in discovery-based proteomics. Accordingly, iTRAQ has become a major tool for both qualitative and quantitative protein researches. In the present study, by comparing the relative expression patterns (in M3 versus M1 leaf samples) of the proteins in the third main-stem leaves, 195 DAPs were identified. Of these 195 DAPs, 91 were up-regulated and 104 were down-regulated. Through the KEGG analysis of DAPs, the following key signaling pathways were identified as significantly enriched for involvement in leaf senescence: metabolic

**Fig. 2** Identification and analysis of differentially abundant proteins from the third main-stem leaves collected from cotton plants grown in field plots. **a** Peptide length distribution based on the iTRAQ proteomic analysis. **b** Bar graph showing the variation in protein coverage

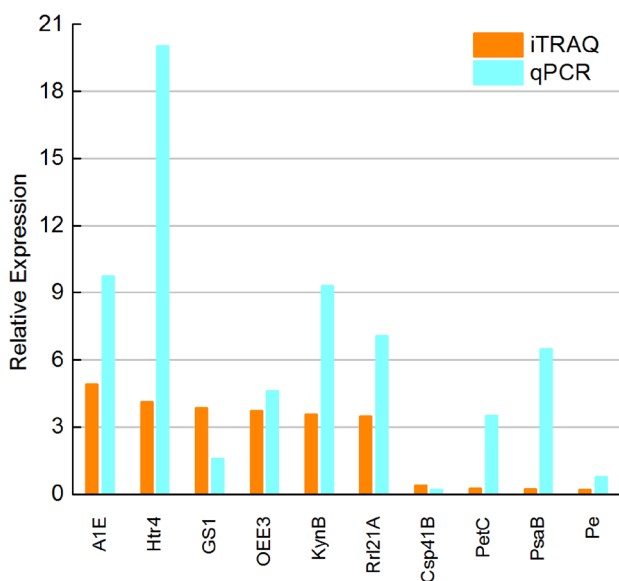


**Fig. 3** Gene Ontology classification of all identified proteins. **a** Number of proteins in each classification of biological process. **b** Number of proteins in each classification of cellular component. **c** Number of proteins in each classification of molecular function



**Table 1** Pathway enrichment analyses of DAPs

Pathway ID	Pathway	Sample1 (118)	<i>p</i> value
ko01100	Metabolic pathways	63	0.00008099
ko00195	Photosynthesis	10	0.00134701
ko00620	Pyruvate metabolism	8	0.02338801
ko00910	Nitrogen metabolism	5	0.03406363
ko00904	Diterpenoid biosynthesis	4	0.00009065
ko00906	Carotenoid biosynthesis	4	0.02097169
ko00052	Galactose metabolism	4	0.04169825
ko00100	Steroid biosynthesis	3	0.00066143
ko05322	Systemic lupus erythematosus	3	0.00337756
ko00600	Sphingolipid metabolism	2	0.03145092
ko04011	MAPK signaling pathway—yeast	2	0.03145092
ko00909	Sesquiterpenoid biosynthesis	1	0.04162257



**Fig. 4** Comparative analysis of RNA and protein levels of selected differentially accumulated proteins as revealed by qPCR and iTRAQ. The wild-type control data are represented by a relative expression value of 1.0. Values are mean  $\pm$  SD ( $n=3$ ) in *cde1* leaf data. *A1E* Aldose 1-epimerase, *Htr4*, Histone H3.3, *GS1* glutamine synthetase nodule isozyme-like, *OEE3* Oxygen-evolving enhancer 3, chloroplastic, *KynB* Kynurenine formamidase, *Rrl21A* 60S ribosomal protein L21-1, *Csp41B* PREDICTED: chloroplast stem-loop binding protein of 41 kDa b, chloroplastic, *PetC* cytochrome b6/f complex subunit IV (chloroplast), *PsaB* photosystem I P700 apoprotein A2 (chloroplast), *Pe* pectinesterase

pathways, photosynthesis, pyruvate metabolism, and nitrogen metabolism.

### Reduced Chl biosynthetic protein levels underlie Chl deficiency in leaves

Leaves are the primary photosynthetic organ of plants (Evans and Von 2011; Makino 2011). Although a critical

and likely adaptive phenomenon, leaf senescence may limit yields and biomass accumulations owing to its associated reduction in photosynthesis. The pigment chlorophyll plays an important role in photosynthesis due to its effects on light capture and energy transfer (Pu et al. 2015). As such, it is vital to the growth of plants and thus crop yield. The present study showed that the Chl *a + b* content of M3 leaves was significantly reduced relative to M1 leaves.

Magnesium chelatase (or Mg-protoporphyrin IX chelatase) is an enzyme that catalyzes the magnesium insertion step of Chl synthesis and consists of three subunits (ChlH, ChlD, and ChII). We observed consistent reductions of some important Chl biosynthesis-related proteins in the M3-stage leaves, and the magnesium-chelatase subunit chlI (ChII) levels were also significantly reduced; the fold change of ChII between the M3 and M1 stages was 0.574.

This protein is part of the metabolism of porphyrin-containing compounds and is involved in the chlorophyll biosynthesis pathway. ChII is also a subunit of Mg chelatase, mainly responsible for redox regulation, and its decrease leads to a decrease in subsequent Chl biosynthesis. This protein is involved in the chlorophyll biosynthesis pathway, which is part of porphyrin-containing compound metabolism. ChII is also the primary redox-regulated subunit of Mg chelatase, and its decreases cause an eventual subsequent decrease in Chl biosynthesis. Similar results were obtained in a study of chloroplast deficiencies in rice (Zhang et al. 2006). Overall, leaf senescence is associated with the decay of chlorophyll synthesis components, which thus result in the failure to effectively synthesize chlorophyll and thereby reduces chlorophyll content over time.

### Downregulation of the photosynthesis pathway

Senescence is an extremely complex biochemical process involving the expression of a number of aging-related genes and signaling pathways (Breeze et al. 2011; van der

Graaff et al. 2006; Shah et al. 2013). We observed that photosynthetic capacity was diminished over the course of senescence. Our study identified ten proteins in the photosynthesis pathway, one of the most important pathways associated with senescence, and most photosystem I (PSI) and photosystem II (PSII) proteins were downregulated by 0.5–1.6-fold.

In addition, five of these proteins were in the PSII complex: PsbB, PsbD, PsbO, PsbP, and PsbQ. PsbB encodes the D2 protein in the PSII reaction center. D2 and D1 constitute two heterologous dimers involved in the electron transfer of most cofactors and pigment molecules. The two proteins are localized within the thylakoid membrane, and their expression was downregulated over the course of leaf senescence. Inhibition of light capture by leaves in photosynthesis resulted in the inhibition of the expression of these proteins. PsbO, PsbP, and PsbQ constitute the oxygen-releasing complexes in higher plants, which collectively play an important role as stabilizing proteins in the oxygen-releasing activity of PSII. In this study, PsbO, PsbP, and PsbQ were upregulated during the aging process, which may indicate that these three proteins protect against the photosynthetic oxygen release system during aging, thus ensuring the absorption of light energy. PsaA (photosystem IP700 apoprotein A1) and PsaB (photosystem IP700 apoprotein A2) are concentrated in the PSI complex, and as leaves aged, their expression decreased as well. This phenomenon indicates that photosynthetic electron transport in senescent leaves is gradually inhibited, thus decreasing photosynthesis. Cytb6 f performs electron transfer between PSII and PSI light responsive complexes, which together modulate the state transition of the photosystem by activating a protein kinase (Hu et al. 2011). Pet D (cytochrome, b6/f, complex subunit IV) and PetA (cytochrome, f) within Cytb6 f are both downregulated, thus restricting electron transport. Overall, senescence impacts the capture of light energy and decreases electron transport function, thus decreasing the photosynthetic rate.

### Nitrogen metabolism pathway

Nitrogen cycling in leaves can cause chlorophyll degradation, thus decreasing photophosphorylation and photosynthesis while inducing leaf senescence (Zhou et al. 2010). In addition, glutamine synthetase (GS) increases in association with leaf senescence (Yin and Chai 1997). In this study, the expression of GS increased as the leaves aged, suggesting that the high expression of GS may increase the nitrogen use of crops while reducing nitrogen loss (Yu et al. 2014). Glutamate synthase (GOGAT) is the key enzyme involved in the assimilation of  $\text{NO}_3^-$ , and its expression decreased in this study. Increases in GS expression and decreases in GOGAT expression both induce excessive glutamine production in plant. Accordingly, as glutamine is involved in

nitrogen transport, the free movement of nitrogen increases throughout leaf senescence.

### Pyruvate metabolism pathway

Pyruvate, a metabolite produced by the glycolysis of cytoplasmic glucose, plays an important role in cell metabolism. Examination of the pyruvate metabolism pathway revealed that six proteins related to energy metabolism were downregulated, while two proteins related to reproductive process and cell apoptosis were upregulated.

### Sphingolipid metabolism pathway

Sphingolipid metabolism is associated with apoptosis (Chipuk et al. 2012). In this study, the expression of two proteins,  $\beta$ -galactosidase ( $\beta$ -Gal, EC3.2.1.23) and  $\alpha$ -galactosidase ( $\alpha$ -Gal, EC3.2.1.22), changed throughout leaf senescence.  $\beta$ -Gal is an enzyme related to cell-wall degradation. The removal of  $\beta$ -galactosidase residues from the non-reductive end of its branched chain can degrade pectin and hemicelluloses as well as glycoproteins and glycolipids. This destabilizes some cell-wall components, causing the cell wall to swell and soften. These changes both release stored energy for the rapid growth and development of plants as well as promote cell-wall degradation during senescence, causing the hydrolysis of polysaccharides, glycoproteins, and galactose residues at the ends of galactose lipids during polysaccharide and cell-wall component metabolism, thus releasing free galactose. The downregulation of  $\beta$ -Gal expression may reduce energy storage during plant growth and development, while  $\alpha$ -Gal is essential for promoting seed germination and maturation, leaf development and senescence, and fruit development and maturation (Sun et al. 2015). In this study, over the course of senescence, the expression of  $\alpha$ -Gal was upregulated, suggesting that  $\alpha$ -Gal promotes leaf senescence.

### Conclusions

In summary, the iTRAQ comparative proteomics approach was used to identify 195 DAPs between senesced leaves (44 day) relative to mature leaves (18 day). Of these, 91 proteins (47%) were upregulated, while the remaining 104 (53%) were downregulated. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses indicated that the enrichment of several pathways potentially associated with aging changed throughout senescence, including metabolic pathways, photosynthesis, pyruvate metabolism, nitrogen metabolism, and diterpenoid biosynthesis. These results may provide a deeper understanding of aging in plants as well as fundamental data describing leaf

senescence in cotton. Further genetic functional analyses are needed to elucidate the molecular mechanisms that regulate chlorophyll metabolism and photosynthesis throughout leaf senescence.

**Author contribution statement** Conceived and designed the experiments: LLT, LCD, and BZY. Performed the experiments: LAC, CHJ, and WM. Analyzed the data: ZYJ, SHCH, WXD, and LYCH. Wrote the paper: LLT.

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