iTRAQ-based quantitative proteomic of tobacco (*Nicotiana tabacum* L.) identified major host proteins involved in photosystems throughout the aging process

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Abstract

Background

Leaf senescence is one of the most common manifestations in plant senescence and has an important effect on photosynthesis. However, the molecular regulation of leaf senescence in response to photosynthesis is still poorly understood. To gain insight into the molecular mechanisms underpinning tobacco, we integrated photosynthesis, organelle ultrastructural, and proteomic analyses of tobacco leaves during senescence.

Results

The photosynthetic rate, intercellular CO₂ concentration, stomatal conductance, transpiration rate showed a downward trend and the stability of organelle decreased with the increasing of tobacco leaves age. iTRAQ and PRM verification were used to analyze the proteins expressed in different periods based on photosynthetic physiology and ultramicroscopic observation. A total of 321, 319, 223 differentially expressed proteins (DEPs) were identified from over maturity (OM) vs immature (IM), OM vs well maturity (WM) and WM vs IM, respectively, including 122/199, 124/195 and 125/98 up/down proteins, respectively. KEGG analysis of DEPs was significantly enriched in metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, starch and sucrose metabolism. In addition, down-regulated proteins were also involved in metabolic pathways such as carbon sequestration of photosynthetic organisms and photosynthesis. Furthermore, PRM analysis indicated that iTRAQ is highly reliable.

Conclusions

Our study provided important technical references for screening photosynthetic host proteins of tobacco leaf senescence and revealing the molecular mechanism during the senescence of tobacco leaves.

1 Background

Leaf senescence is one of the most common manifestations of plant senescence and chloroplasts are the main sites for photosynthesis in plants [1, 2]. During leaf senescence, chlorophyll degradation is one of the most important catabolism, and it is one of the main causes of leaf yellowing during plant senescence. Chlorophyll content is often used to characterize plant senescence [3, 4]. Simultaneously, large molecules such as proteins and nucleic acids are degraded, which causes a decline in photosynthesis during leaf senescence. Therefore, leaf senescence has an important effect on photosynthesis [5, 6].

Plant senescence is regulated by gene and affected by environment [7, 8]. Yan et al. studied the phenotype of transgenic plants and mutants of *Arabidopsis* using reverse genetics methods and found that AtSPX 1 can accelerate the senescence of *Arabidopsis* leaves, but the senescence of mutant plants

is not obvious [9]. Christiansen et al. found that HvNA005 gene expression was up-regulated after treating wheat with ABA, indicating that HvNA005 is an obvious target for fine-tuning gene expression to promote wheat senescence-related nutrient reuse [10]. Gao et al. identified 113 B3 proteins from tobacco by comparative genomic analysis and divided them into 4 subfamilies of ARF, RAV, LAV, and REM. After analyzing the B3 protein of tobacco, they found that the REM family genes were up-regulated in response to topping, while ARF is down-regulated [11]. However, previous studies mainly focused on reflecting the regulation mechanism at the transcription level, and the molecular regulation mechanism of photosynthesis in response to plant senescence from the protein expression level has rarely been reported.

Tobacco is one of the classic model plants, and studies on the senescence mechanism of tobacco leaves have certain guiding significance for the research on the senescence mechanism of other crops [12]. Tobacco is an important economic crop. During the cultivation process of flue-cured tobacco, there is a technical measure of topping and wiping off the branches, which has changed the law of material accumulation in the leaves. Therefore, it has also changed the normal senescence pattern of tobacco leaves [13]. During the harvest process, the maturity of the tobacco leaves is often judged based on the appearance characteristics of leaves, including color, fluff, and veins, which are greatly affected by the subjective influence of harvesters, and there are major defects in the determination of the maturity of tobacco leaves [14]. The difference in the quality of fresh tobacco leaves cause problems such as difficulty in curing tobacco leaves and poor quality after curing. Therefore, analyzing the molecular mechanism of photosynthesis response in tobacco leaf senescence and screening host proteins for photosynthesis in tobacco leaf senescence are of great significance for the accurate judgment of plant and tobacco senescence.

Proteomics is an important part of systems biology, which is widely used to study the expression of proteins such as growth and development, mutation and environmental changes in plants and animals. Proteomics can screen potential protein targets from DEPs [15, 16]. High-throughput and high-resolution protein isolation and identification techniques are commonly used for quantitative and qualitative analysis of proteins [17]. The iTRAQ for protein quantitative analysis has the characteristics of high sensitivity, strong separation ability, wide application range, high throughput, reliable results and high automation [18]. Targeted proteomics is used to quantify the target protein and peptide segments for the selected, interested proteins or peptides, and to verify specific scientific hypotheses, such as SRM technology and PRM technology [19, 20].

With the continuous improvement of proteomics technology, proteomics is gradually applied to the research of tobacco proteomics. For example, Das et al. used iTRAQ technology to perform protein expression analysis on infected TMV and mock infected tobacco plants [21]. A total of 407 differential proteins were identified, and several major host proteins related to TMV infection / replication were found. Most of the tobacco proteomics research involved in resistance to stress, pests and diseases [22, 23]. Proteomics studies on protein changes in tobacco leaves during the senescence, especially those related to photosynthesis are scarce.

The purpose of this study is to analyze the protein expression on tobacco variety K326 at different stages by using iTRAQ technology. PRM technology was used to verify the expression of differentially expressed proteins to screen out the target proteins of tobacco leaf senescence and verify the reliability of iTRAQ results. By analyzing internal mechanism of tobacco leaves which represents the color changes of tobacco leaves during tobacco senescence, so that to discover the proteins closely related to tobacco senescence. This study can not only scientifically explain the senescence mechanism of tobacco leaves, but also provide a theoretical basis for the subsequent development of a new method to quickly and accurately determine the maturity of tobacco leaves.

2 Results

2.1 Photosynthesis at Different Maturity Stages

During tobacco senescence, photosynthesis of tobacco leaves at three different maturity stages was studied. The photosynthetic rate at IM, WM and OM were $15.00 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$, $11.50\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$ and $5.90\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$, respectively. The photosynthetic rate of tobacco leaves at IM and WM was significantly higher than that at OM, and there was no significant difference between IM stage and WM stage (Fig. 1A). The concentration of intercellular CO_2 in tobacco leaves at IM, WM and OM were $510 \mu\text{mol}\cdot\text{mol}^{-1}$, $400 \mu\text{mol}\cdot\text{mol}^{-1}$ and $280 \mu\text{mol}\cdot\text{mol}^{-1}$, respectively. The intercellular CO_2 concentration of tobacco leaves in IM and WM periods was significantly higher than that in OM periods, and there was no significant difference in intercellular CO_2 concentration between IM and WM periods (Fig. 1B). The stomatal conductance of tobacco leaves at IM WM and OM were $0.24 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, $0.13 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $0.11 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively. The stomatal conductance of tobacco leaves in IM was significantly higher than that in WM and IM, and there was no significant difference between IM and OM (Fig. 1C). The stomatal conductance of tobacco leaves at IM, WM and OM was $2.75 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, $1.40 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $1.30 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively. The transpiration rate of tobacco leaves in IM was significantly higher than that in WM and OM, and there was no significant difference between WM and OM (Fig. 1D). In conclusion, photosynthetic rate, intercellular CO_2 concentration, stomatal conductance and transpiration rate decreased with increasing of leaf age.

2.2 Chloroplasts configuration and ultrastructure

By observing the configuration and ultrastructure of chloroplasts, it was found that the IM leaves had orderly arrangement of chloroplasts, and complete chloroplast membrane and less starch granules (Fig. 2A). After enlargement, the proportion of starch granules in chloroplasts was small, and the chloroplast structure was complete, the cell wall structure was incomplete, and the grana lamellae were closely arranged (Fig. 2B). The structure of chloroplasts in WM leaves was similar to that in IM, in addition, starch granules were clearly visible, the number of starch granules increased obviously and the volume increased in WM leaves (Fig. 2C). After enlargement, the arrangement of grana lamellae was loose, the gap was enlarged, and the structure of cell wall was clear, and the structure of chloroplast

membrane was complete (Fig. 2D). During the OM stage, the number of chloroplasts decreased significantly, and the chloroplast membrane dissolved gradually, the volume and the number of starch granules were increased. With the dissolution of chloroplast membrane, the chloroplasts were exposed to cells (Fig. 2E). After enlargement, almost all chloroplasts contained a certain amount of starch granules, and the number of grana lamellae decreased compared with the WM. The cell wall was also blurred (Fig. 2F). Thus, with the increasing of leaf age, the number of chloroplasts decreased, and the grana lamella became loose gradually from compact arrangement. The number of grana lamellae increased firstly and then decreased. The chloroplast membrane also gradually dissolved. The proportion of starch granules was positively correlated with increasing of leaf age.

2.3 Identification of Proteins at Different Maturity Stages

The number of secondary spectra produced by mass spectrometry and the number of secondary spectra analyzed for three groups of tobacco leaves at different growth stages (IM, OM and WM) were 435604 and 158095, respectively. The identification rate of spectra was more than 36.29%. The number of identified peptide segments was 23346, and the number of identified proteins was 4747. The number of identified proteins containing at least two specific peptide segments was 3292, which accounted for 69.35% of total protein amount.

2.4 Quantitative Analysis of Protein between Samples

If there is no significant change in the amount of the same protein between the two samples in relative quantification, the protein abundance ratio is close to 1. Pairwise comparisons of proteins with P-values < 0.05 and fold-changes > 1.2 or < 0.83 in abundance were regarded as differentially regulated proteins (DRPs). We identified a total of 321 DRPs, including 122 up-regulated and 199 down-regulated proteins, by comparing the OM against the IM (Fig. 3A). In contrast, we obtained 319 DRPs, including 124 up-regulated and 195 down-regulated proteins, by comparing the OM against the WM (Fig. 3B). There were 223 DRPs, including 125 up-regulated and 98 down-regulated proteins between WM and IM (Fig. 3C). Figure 4 was a Venn diagram showing significant differences in protein expression between pairwise comparisons. Further comparisons of different proteins between the control groups revealed that there were 86 differentially expressed proteins among OM vs IM, OM vs WM and WM vs IM of the three control groups.

2.5 KEGG Metabolic Pathway of Differentially Expressed Proteins

To further investigate the biological functions of these DEPs, KEGG pathway analysis was performed using the BLAST v2.2.26 (NCBI) program. Four pathways were significantly enriched among the DEPs of OM vs IM, OM vs WM and WM vs IM, including “metabolic pathways”, “Biosynthesis of secondary metabolites”, “Microbial metabolism in diverse environment” and “Starch and sucrose metabolism”. By comparing the OM against the IM, a total of 237 DEPs (74%) were mapped to 79 pathways in the KEGG database (Table S1). Figure 5 (A) was a statistical pie chart of Pathway function, which ranked the top ten in the list of up and down-regulation differential proteins between over-mature (OM) and immature

(IM) tobacco leaves. As can be seen from the figure, Pathway metabolic function types in the top 10 were different in all up-regulation and down-regulation differential proteins. The Pathway functional annotations of the two groups have the same four functions in the top 10; We identified 123 proteins (30 proteins were up-regulated and 93 proteins were down-regulated) that belong to "metabolic pathways", 55 proteins (22 proteins were up-regulated and 33 proteins were down-regulated) that belong to "Biosynthesis of secondary metabolites", 40 proteins (8 proteins were up-regulated and 32 proteins were down-regulated) that belong to "Microbial metabolism in diverse environment", 14 proteins (7 proteins were up-regulated and 7 proteins were down-regulated) that belong to "Starch and sucrose metabolism". In addition, up-regulated proteins were also involved in metabolic pathways such as "Protein processing in endoplasmic reticulum" (8), "Tyrosine metabolism" (7), "Phenylpropanoid biosynthesis" (6), "ABC transporters" (5) and "Plant-pathogen interaction" (5) and "Isoquinoline alkaloid biosynthesis" (5). Down-regulation of protein involves in "Photosynthesis" (26), "Carbon fixation in photosynthetic organisms" (18), "Glycolysis/glycogenesis" (11), "Ribosome" (8), "Oxidative phosphorylation" (7), "Pentose phosphate pathway" (7) and so on (Fig. 5A).

By comparing the OM against the WM, a total of 237 DEPs (74%) were mapped to 82 pathways in the KEGG database (Table S2). We identified 126 proteins (34 proteins were up-regulated and 92 proteins were down-regulated) that belong to "metabolic pathways", 64 proteins (28 proteins were up-regulated and 36 proteins were down-regulated) that belong to "Biosynthesis of secondary metabolites", 37 proteins (8 proteins were up-regulated and 29 proteins were down-regulated) that belong to "Microbial metabolism in diverse environment", 19 proteins (8 proteins were up-regulated and 11 proteins were down-regulated) that belong to "Starch and sucrose metabolism". Up-regulated proteins were also involved in "Phenylpropanoid biosynthesis" (8), "Tyrosine metabolism" (8), "Phenylalanine metabolism" (7), "Isoquinoline alkaloid biosynthesis" (6), "Splicer" (6) and "Protein processing in endoplasmic reticulum" (9), which were different from the functional metabolic pathways of down-regulated proteins. Down-regulated proteins were involved in metabolic pathways, including "Photosynthesis" (25), "Carbon fixation in photosynthetic organisms" (13), "Glycolysis/glycogenesis" (9), "Pentose phosphate pathway" (8), "Glutathione metabolism" (7) and "Oxidative phosphorylation" (7) and so on (Fig. 5B).

By comparing the WM against the IM, a total of 163 DEPs (73%) were mapped to 78 pathways in the KEGG database (Table S3). We identified 79 proteins (39 proteins were up-regulated and 40 proteins were down-regulated) that belong to "metabolic pathways", 45 proteins (27 proteins were up-regulated and 18 proteins were down-regulated) that belong to "Biosynthesis of secondary metabolites", 26 proteins (10 proteins were up-regulated and 16 proteins were down-regulated) that belong to "Microbial metabolism in diverse environment", 12 proteins (7 proteins were up-regulated and 5 proteins were down-regulated) that belong to "Starch and sucrose metabolism". In addition, up-regulated proteins were also involved in metabolic pathways including "Phenylpropanoid biosynthesis" (6), "Protein processing in endoplasmic reticulum" (6), "Phenylalanine metabolism" (5), "Plant-pathogen interaction" (5), "ABC protein transporters" (4) and "Glutathione metabolism" (4). Down-regulated proteins were involved in metabolic pathways including "Carbon fixation in photosynthetic organisms" (12), "Ribosomes" (8),

“Photosynthesis” (7), “Glycolysis/gluconeogenesis” (5), “Pentose phosphate pathway” (5) and “Fructose and mannose metabolism” (4) and so on (Fig. 5C).

2.6 Thermogram Analysis of Differential Proteins

A total of 86 DEPs from six significantly enriched pathways in the senescence of tobacco leaves are shown with their abundance patterns and functional annotation in Fig. 6. Many DEPs involved in metabolic pathways (32), biosynthesis of secondary metabolites (15), metabolism of microorganisms in different environments (11), carbon sequestration of photosynthetic organisms (10), and some DEPs involved in photosynthesis (8), starch and sucrose metabolism (6) were reduced sharply. In photosynthesis metabolic pathways, there were eight proteins that related to photosynthesis, including A0A140G1P2, A0A1S4DCM1, A0A140G1 × 0, D2K7Z2, A0A140G1R2, A0A1S4CFV4, A0A140G1P8 and A0A140G1S1.

2.7 Photosynthetic proteins expression patterns

Photosynthesis is critical for tobacco leaves yield; hence, we investigated the abundance of proteins involved in photosynthesis pathway. Several DEPs concentrated in photosynthetic pathways and one typical pathway was shown in Fig. 7A. Almost all the photosynthetic DEPs enriched in core parts of photosynthesis, such as photosystems (I and II), cytochrome b6f complex, electron transports, and ATP synthases. Compared OM with IM, there were 23 DEPs involved in photosynthetic pathway, including one up-regulated protein and 22 down-regulated proteins. 24 DEPs involved in photosynthetic pathway were shown in Fig. 7 (B) by comparing OM with WM, including one up-regulated protein and 23 down-regulated proteins. The enrichment of all photosynthetic DEPs was similar to OM vs IM. Compared WM with IM, there were 9 DEPs involved in photosynthetic pathway, including 2 up-regulated proteins and 7 down-regulated proteins which enriched in photosystems (I and II) and ATP synthases (Fig. 7C).

2.8 PRM verification

Three proteins, including A0A1S4DCM1, A0A140G1R2 and A0A140G1P2 which were significantly expressed and related to photosynthesis were selected and their expression was examined by PRM. Skyline can not only visualize the target peptide ion signal which is collected by mass spectrometry, but also judge the quality and content of the peptide signal by observing the peak shape, peak intensity, and retention time consistency [24]. The effect is immediately visible when the ratios are presented in a density plot (Fig. 8A, B and C) or in a PRM plot (Fig. 9A, B and C). Compared with the quantitative results of iTRAQ, three differentially expressed proteins showed the same level (A0A1S4DCM1 was up-regulated and A0A140G1R2 and A0A140G1P2 were down-regulated) of expression which confirmed the reliability of iTRAQ.

3 Discussion

Photosynthetic rate, intercellular CO₂ concentration, stomatal conductance and transpiration rate are comprehensive evaluation indicators of photosynthesis [25]. Photosynthetic rate and intercellular CO₂

concentration in IM and WM were significantly higher than those in OM. Canales et al. cultured sunflower in different concentrations of CO₂, and found that sunflower senescence was delayed in high concentrations of CO₂ [26]. There were researches indicated that higher intercellular CO₂ concentration and stomatal conductance were factors that improved photosynthesis. Photosynthesis was inseparable from photosynthesis rate, intercellular CO₂ concentration, stomatal conductance and transpiration rate [27]. During the senescence of tobacco leaves, the decrease of stomatal conductance resulted in the decrease of gas exchange, which decreased the intercellular CO₂ concentration and transpiration rate, then resulted in the decrease of photosynthetic rate. The decline of photosynthesis related index indicated that the photosynthesis of tobacco leaves decreased significantly during senescence.

The number of chloroplasts showed a downward trend with the increasing of tobacco leaf age. The grana lamellae gradually became loose from close arrangement, and its amount increased first and then decreased, and the chloroplast membrane gradually dissolved. The proportion of starch grains was positively correlated with the increasing of tobacco leaf age, which was consistent with the results of Mayta et al. [28]. There are lots of studies found that sugar was an important signal for regulating plant metabolism and development. The accumulation of sugar in leaves can lead to plant senescence [29]. Starch granules would be accumulated during the process of leaves growth and the chloroplast structure would be broken down when starch granules were accumulated enough [30]. The breaking down of chloroplast structure will weaken the photosynthesis of leaves.

To maximize information on the tobacco proteome, we prepared and identified protein extracts from three stages of tobacco leaves using iTRAQ coupled with LC-MS/MS technology. There were differences among the pairwise comparisons in protein expression, which indicated that the divergences in the appearance of tobacco leaves at various stages were accompanied by protein expression differences. Between OM vs IM and OM vs WM, the number of down-regulated proteins was greater than that of up-regulated proteins, which indicated that the number of protein decreased during tobacco leaves senescence. In the senescence of tobacco leaves, up-regulation proteins may be involved in the material catabolic pathway, while down-regulation proteins were mainly involved in substance anabolic pathway and the metabolic process related to photosynthesis. Zhang et al. [31] found that up-regulated proteins were involved in energy metabolism, and down-regulated proteins were involved in photosynthesis and electron transport by proteomic analysis of rice aging process.

KEGG metabolic pathways analysis of differential proteins was conducted to further understand main biochemical metabolic pathways and signal transduction pathways that DEPs involved in. The DEPs involved in "Metabolic pathways", "Biosynthesis of secondary metabolites" and "Microbial metabolism in diverse environments", "Starch and sucrose metabolism". Ma et al. [32] carried out proteomic studies on four developmental stages of daylily flower. KEGG annotation results showed that DEPs were enriched in starch and sucrose metabolism. Starch accumulated slowly in the early stage and reached the peak at the mature stage. With the increasing of leaf age, starch gradually metabolized and its content showed a downward trend [33]. Secondary metabolism was the metabolic process of nicotine and major aroma

precursor's synthesis and catabolism in plants. Studies have shown that the content of nicotine and reducing sugar in leaves increased with the increasing of tobacco leaf age [34].

Up-regulated proteins were also involved in the metabolic pathways including "Endoplasmic reticulum protein processing" and "Phenylpropanoid biosynthesis". Phenylpropanoid was a kind of secondary metabolite, and its protein expression was up-regulated in the biosynthesis of phenylpropanoid, which further indicated that the biosynthesis process of secondary metabolite increases with the increasing of tobacco leaf age. During the senescence of tobacco leaves up-regulated proteins were involved in endoplasmic reticulum protein processing. Proteins processed by endoplasmic reticulum may be protein degrading enzymes, which sped up the catabolism of proteins and other substances. Zhang et al. [35] found that proteases related to protein degradation, such as cysteine protease, aspartic protease and proteasome subunit alpha type-3, were up-regulated during tobacco leaves senescence, which indicated that the synthesis and catabolism of proteins were simultaneous in the senescence of tobacco leaves. There are some reasons that lead to the decrease of proteins during the senescence of tobacco leaves, including the decrease of protein synthesis rate, the acceleration of degradation rate, and the contact of substrate and hydrolase [36].

The proportion of down-regulated proteins in "Metabolic pathways" and "Microbial metabolism in different environments" was higher than that of up-regulated proteins. Metabolic pathways included anabolism and catabolism. The down-regulation of protein expression related to metabolic pathways and microbial metabolism in different environments indicated that the metabolic ability of tobacco leaves declined during tobacco senescence. Down-regulated proteins were also involved in metabolic pathways including "Photosynthesis", "Carbon fixation in photosynthetic organisms", "Glycolysis and Glycogenesis". There are two stages in Photosynthesis, including light reaction and dark reaction. During light reaction, leaves absorbed light energy to reduce NADP^+ to NADPH and phosphorylate ADP to ATP, which provided energy for carbon assimilation in dark reaction [37, 38, 39]. The decline of protein expression involved in photosynthesis, carbon sequestration of photosynthetic organisms and glycolysis and glycogen regeneration indicated that photosynthesis and sugar synthesis were weakened during tobacco leaves senescence. Plaxton et al. [40] found that plant glycolytic enzymes were multifunctional proteins involved in the process, rather than carbohydrate metabolism. ATP was produced in glycolysis pathway [41], the down-regulation of protein expression related to glycolysis and glycogenesis indicated that ATP synthesis was decreased and sugar synthesis weakened during tobacco leaves senescence.

According to the enrichment of 86 proteins, they were mainly involved in metabolic pathways, including metabolic pathways, biosynthesis of secondary metabolites, metabolism of microorganisms in different environments, carbon sequestration of photosynthetic organisms, photosynthesis and metabolism of starch and sucrose. Photosystem I and II were photosynthetic electron transport centers [42]. According to the search results of 8 proteins related to photosynthesis, 3 proteins with significant differences between the comparison groups were screened for PRM verification. In the PRM validation process, three differentially expressed proteins were consistent with the quantitative results of iTRAQ in pairwise comparisons. Therefore, three differentially expressed proteins including A0A1S4DCM1, A0A140G1R2

and A0A140G1P2 were likely to be key regulators of tobacco leaves senescence, and can be used as marker protein for tobacco leaves senescence.

Photoreaction of photosynthesis is driven by protein complexes such as photosystem I, photosystem II, cytochrome b6/f complex, ATP synthase, and so on [43]. Oxygen-evolving enhancer protein 3 – 2, chloroplastic-like was a protein related to photosystem II, which participated in oxygen release during photosynthesis and maintained the stability of photosynthesis system [44, 45, 46]. In this study, the expression of A0A1S4DCM1 was up-regulated during tobacco leaves senescence, which was consistent with previous research results [47, 48, 49]. It is indicated that during the senescence of tobacco leaves, attributing to the decline of photosynthesis, the A0A1S4DCM1 is increased in order to maintain the stability of the photosynthetic system. In addition, oxygen-evolving enhancer proteins are products of degradation of the photosynthetic system, the oxygen-evolving enhancer protein increased with the degradation of the photosynthetic system [50]. A0A140G1R2 (Photosystem I P700 chlorophyll a apoproteinA2) is an important protein for chloroplasts to maintain normal physiological functions [51, 52]. In the senescence of tobacco leaves, the decrease of A0A140G1R2 indicates that photosynthetic electron transport in senescent leaves is gradually inhibited, thus decreasing photosynthesis. A0A140G1P2 (Photosystem II protein D1) was the core subunit of photosystem II, which played an important role in maintaining the stability of photoreaction center system II. It also provided binding sites for electron-transfer oxidative coenzyme factors and involved in the electron transfer of most cofactors and pigment molecules [53]. The down-regulation of PSII protein D1 indicated that the transmission of electronic capabilities and the stability of PSII were decreased, and the function of PSII was weakened.

4 Conclusion

In this study, an overview of the protein expression in tobacco leaves to response to senescence was first explored by iTRAQ technique. Tobacco leaves showed significant changes in the photosynthetic rate, intercellular CO₂ concentration, stomatal conductance, transpiration rate and organelle ultrastructural. These physiological changes not only revealed the tobacco leaves' ripening metabolism but provided an important link with dynamic protein changes obtained using iTRAQ. This large-scale proteomic study as a hypothesis-free approach provides quite a complete view of the major and important pathways which evolve during tobacco leaves development, thus providing a better understanding of tobacco leaves development and ripening physiology. These findings help provide an understanding of the “Metabolic pathways”, “Biosynthesis of secondary metabolites” and “Microbial metabolism in diverse environments”, “Starch and sucrose metabolism” and “Photosynthesis” of tobacco leaves which all largely determine final tobacco leaves quality. Finally, some proteins underwent major changes at specific developmental stages; thus, they can be used as novel protein biomarkers of tobacco leaves development, which have not been detected to date. Consequently, this may help open up new lines to explore the parameters controlling tobacco leaves development and ripening.

5 Methods

5.1 Plant Materials

The seeds of the tobacco variety K326 were acquired from Zhongyan Tobacco Seed Co., Ltd, China. Tobacco seedlings (*Nicotiana tabacum* L.) were transplanted to potting mixture. The seedlings were grown from April to August 2018 under typical Yunnan climate conditions. Healthy tobacco plants of 3 periods, IM leaves (70 days after transplanting), WM leaves (80 days after transplanting) and OM leaves (90 days after transplanting) were separately collected with two biological replicates and then stored at -80°C immediately until sent to Wuhan Jinkairui Bioengineering Co., Ltd. for testing (Fig. 10).

5.2 Determination of Photosynthetic Rate

LI-6400 portable photosynthetic system (Li-Cor Inc., Lincoln, NE, USA) was used to measure the photosynthetic indexes of tobacco leaves at different maturity stages [54]. The photosynthetic rate, intercellular carbon dioxide concentration, stomatal conductance and transpiration rate of tobacco leaves were measured at IM, WM and OM stages respectively. The determination time was once every two hours between 8:00–18:00, and five times in total, and calculated the average value. During the measurements, the leaf temperature was adjusted to 25°C . Sigma Plot is used to plot according to the data.

5.3 Electron Microscope Scanning

Refer to Li et al.'s [55] methods with slight modification. Fresh tobacco leaves with different maturity IM, WM and OM were to be cut into $3\text{ mm} \times 3\text{ mm}$ size leaves, and avoid the main vein and large lateral vein of leaves, then placed in a penicillin bottle, and immersed the leaves in 4% glutaraldehyde fixing solution. The leaves were fully immersed in fixing solution by syringe extraction. The samples were fixed by 1% OsO_4 (osmium tetroxide) for 2 hours, and dehydrated by acetone, and embedded by epoxy resin (spi-812) for 24 hours, and sliced (German Leica uc6) into thin slices. The slices were stained with lead citrate and uranyl acetate and were observed and photographed under Hitachi-A-2 transmission electron microscope.

5.4 Extraction of total proteins from tobacco leaves

Middle tobacco leaves of three periods were harvested and ground with a tissue homogenizer, in TissueLyzer LT (Qiagen) with liquid nitrogen until the leaves became fine powder. The ground powder was resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% SDS (sodium salt), 40 mM Tris-Cl, pH 8.5, 1 mM PMSF (phenylmethanesulfonyl fluoride), 2 mM EDTA (ethylene diamine tetraacetic acid)) for 5 min. The suspension was added 10 mM DL-Dithiothreitol (DTT) and placed in an ultrasonic cell disruptor (JY92-11N, Ningbo, China) sonicated for 15 min in an ice bath, then centrifuged at 13000 g , 4°C for 20 minutes in a desktop high speed refrigerated centrifuge (TGL-20M, Hunan, China). After that, transferred the supernatant to a new centrifugal tube, and added cold acetone with a volume of 4 times the supernatant to the centrifugal tube, which was placed overnight at -20°C .

Collected the protein precipitation by centrifugation and dried in air. Added 8M urea/100 mM Borane-triethylamine complex (TEAB, pH 8.0) solution to redissolve the protein. Then added DL-Dithiothreitol till the final concentration was 10 mM, and the reduction reaction was carried out in water bath at 56°C for

30 minutes. After that, added Iodoacetamide (IAM) till the final concentration was 55 mM and the alkylation reaction was carried out at dark in room temperature for 30 minutes. The protein concentration was determined by Bradford method.

5.5 Separation and Identification of Proteins

Protein extraction and separation, iTRAQ quantification, bioinformatics analysis and PRM validation were carried out according to the following procedures (Fig. 11).

5.5.1 Enzymatic Hydrolysis and Desalination

For each sample, 100 µg of protein was taken for trypsin digestion and then diluted 5 times with 100 mM Triethylamine borane (TEAB). Trypsin was added at a mass ratio of 1:50 (trypsin: protein), and then enzymatic hydrolysis was digested overnight at 37 °C. After enzymatic hydrolysis, the peptide segments were desalted by AAA-C18 column (150 mm × 4.6 mm I.D., 5! M, AB SCIEX), and then frozen and dried in vacuum.

5.5.2 iTRAQ labeling

Dissolved the Peptides with 0.5M TEAB and labeled with iTRAQ-8 kit (AB SCIEX, USA) according to the manufacturer's instructions, Six samples were labeled as follows: IM-1, IM-2, WM-1, WM-2, OM-1, OM-2 were labeled with iTRAQ tag 115, iTRAQ tag 116, iTRAQ tag 117, iTRAQ tag 118, iTRAQ tag 119 and iTRAQ tag 121. All of the labeled samples were mixed with equal amount. All of the labeled peptides were mixed. Then the mixed samples were fractionated using a high-performance liquid chromatography (HPLC) system (Thermo DINOEX Ultimate 3000 BioRS, USA) using a Durashell C18 (5 µm, 100 Å, 4.6 × 250 mm). A total of 42 secondary fractions were collected and merged into 12 fractions for analysis. The fractions were then desalinated on the Strata-X C18 column (Phenomenex, Torrance, USA) and dried in vacuum.

5.5.3 LC-MS/MS Analysis

LC-MS/MS analysis was performed on Triple TOF 5600 plus mass spectrometer (AB SCIEX, Massachusetts, USA). Dissolved the polypeptide samples in 2% acetonitrile/0.1% formic acid and analyzed by Triple TOF 5600 plus mass spectrometer coupled with Eksigent nanoLC system (SCIEX, USA). The polypeptide solution was added to the C18 capture column (5 µm, 100 µm × 20 mm), and gradient elution was carried out on the C18 analytical column (3 µm, 75 µm × 150 mm) at a 90-minute time gradient and 300 nL/min flow rate. The two mobile phases are respectively mobile phase A (2% acetonitrile / 0.1% formic acid / 98% H₂O) and mobile phase B (98% acetonitrile / 0.1% formic acid/2% H₂O). For information dependent acquisition (IDA), the first-order mass spectrogram was scanned with 250 m/s ion accumulation time, and the second-order mass spectrogram of 30 precursor ions was collected with 50 ms ion accumulation time. The MS1 spectra was collected in the range of 350-1500m/z, and the MS2 spectra was collected in the range of 100-1500m/z. The precursor ions were excluded from reselection for 15 s.

5.5.4 Protein Identification and data analysis

The original MS/MS data were submitted to ProteinPilot Software v4.5 (Applied Biosystem, USA) for data analysis. Protein identification was performed searching against *Triticum aestivum* database ([www.uniprot.org/Triticum aestivum](http://www.uniprot.org/Triticum%20aestivum) (wheat)) using Paragon algorithm. ProteinPilot search parameters are shown in Table 1. Unused Prot-Score ≥ 1.3 (95%) as threshold with at least one peptide above the 95% confidence was considered as benchmark for protein identification.

Table 1
ProteinPilot searching parameters

Item	Value
Type of search	iTRAQ 8plex (Peptide Labeled)
Enzyme	Trypsin
Cys Alkylation	Iodoacetamide
Instrument	TripleTOF 5600
Bias Correction	TRUE
Background Correction	TRUE
ID focus	Biological modifications
Search Effort	Thorough ID
Protein Mass	Unrestricted
Database	uniprot-taxonomy%3A-Nicotiana + tabacum.fasta(0 sequence in total)

For false discovery rate (FDR) calculation, an automatic decoy database search strategy [56] was employed to estimate FDR using the PSPEP (Proteomics System Performance Evaluation Pipeline Software, integrated in the ProteinPilot Software). All reported proteins were based on the following conditions: (1) at least one peptide confidence > 95% (unused Prot Score ≥ 1.3); (2) FDR < 1%. The tobacco leaves samples with different maturity were compared between two (OM vs IM; OM vs WM; WM vs IM). Under the condition of difference multiple ≥ 1.2 or ≤ 0.83 times and P value ≤ 0.05 , they could be considered to be significantly expressed proteins [57].

ProteinPilot search parameters are shown in Table 1:

5.6 Bioinformatics Analysis

Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>) was used to annotate differentially expressed proteins to understand their biological functions. All other pictures in this paper

were drawn with R language (<http://www.r-project.org/>). All data related to this study has been public available on iProX (www.iprox.org) with ID IPX0001668000/ IPX0001668001/ IPX0001668002.

5.7 PRM Quantitation Analysis

Protein preparation was performed as described above. Targeted MS analysis using PRM (parallel reaction monitoring) was performed on a TripleTOF 5600 LC-MS/MS system (AB SCIEX, Massachusetts, USA). For PRM data collection, the list of optimized protein peptides is added to the inclusion list, and the mass spectrometer selects the size of the peptides in the list one by one to be broken and collects the secondary ion spectrum. All the peptide samples obtained by enzymatic hydrolysis were firstly mixed and then subjected to mass spectrometry DDA (Data Dependent Acquisition) detection. The ProteinPilot database was searched, and then the Skyline software was introduced to establish a spectrum library, and the peptides of the target protein were screened for method establishment. Design the PRM detection method and add the target peptide m/z to the inclusion list to establish a mass spectrometry acquisition method. The PRM method is then used to perform PRM data acquisition on the mixed samples to adjust and optimize the PRM acquisition method to form a final PRM method for sample data collection. Each sample was dissolved with Loading Buffer (0.1% formic acid/3% acetonitrile), and the supernatant was spotted after centrifugation. Data acquisition was performed on each sample using the optimized PRM mass spectrometry method described above to obtain 2 PRM spectral files. By extracting and analyzing these PRM spectrum files, quantitative information of the protein can be obtained. By extracting and analyzing these PRM spectrum files, quantitative information of the protein can be obtained.

5.8 Statistical analysis

Analyses of the statistical differences in protein expression levels between samples were performed using SPSS 21.0 statistical software (IBM, Armonk, NY, USA). All data in this study were presented as means $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine a significant difference between the groups with A 2-tailed p-value < 0.05 was considered to be statistically significant.

Abbreviations

DEPs: differentially expressed proteins; iTRAQ: isobaric tag for relative and absolute quantification; PRM: parallel reaction monitoring; OM: over maturity; WM: well maturity; IM: immature; KEGG: [Kyoto Encyclopedia of Genes and Genomes](#); DRPs: differentially regulated proteins;

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. All authors approved the final manuscript.

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Authors' Contributions

ZL and CZ conceived the original research plans. GZ and CH designed the experiments. PL and TX performed the experiments. YT and SS analyzed the data. YC and CH wrote the manuscript. All authors reviewed and approved the final manuscript.

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Additional Files

Additional file 1: Table S1. Pathway enrichment of differentially expressed proteins between OM and IM

Additional file 2: Table S2. Pathway enrichment of differentially expressed proteins between OM and WM

Additional file 3: Table S3. Pathway enrichment of differentially expressed proteins between WM and IM

Figures

Figure 1

Photosynthesis at different maturity stages. (A) Photosynthetic rate; (B) Inter-cellular CO_2 concentration; (C) Tomatal conductance; (D) Transpiration rate; (*) Represent significant differences at $p < 0.05$.

Figure 2

Mesophyll cell and chloroplast ultrastructure changes in tobacco leaves. The left column (A, C, E) shows the chlorophyll mesophyll cells and topography ($\times 6000$). Shown in the right column (B, D, F) is the ultrastructure ($\times 20,000$) of mesophyll cells and chloroplast. Micrographs in rows A–B, C–D, and E–F, respectively, were from plants IM, WM and OM. IS, Intercellular space; CW, cell wall; Ch, Chloroplast; S, starch granule; Chm, chloroplast membrane; G, Grana lamellae.

Figure 3

The volcano plot shows the collected p-values for the changes in the patterns of all identified proteins in pairwise comparisons. (A) DEPs in OM vs IM; (B) DEPs in OM vs WM; (C) DEPs in WM vs IM. Black spots are unchanged proteins, Green spots represent down-regulated proteins and the red spots represent up-regulated protein. Blue spots represent candidates with a putative role.

Figure 4

Venn diagram of differentially expressed proteins between different groups

A

B

C

Figure 5

The top ten metabolic pathways involved by three groups of differential proteins. (A) OM vs IM metabolic pathways involved by differential proteins. (B) OM vs WM metabolic pathways involved by differential proteins. (C) WM vs IM metabolic pathways involved by differential proteins.

Figure 6

Differential protein relative expression matrix heatmap of the three groups; the color in the figure indicates the relative expression level of the protein in the sample. The yellow color indicates that the protein has a higher expression level in the sample, and the blue color represents a lower expression level. Color bars represent specific expression abundance.

Figure 7

Representative significantly enriched photosynthesis related KEGG pathways. (A) The OM vs IM significantly enriched photosynthesis pathway; (B) the OM vs WM significantly enriched photosynthesis pathway; (C) the WM vs IM significantly enriched photosynthesis pathway. The abundance of proteins is represented by different colors, with red for up-regulated, green for down-regulated, and black for stable expression of proteins.

Figure 8

Visualization of skyline peptide results. (A) The ratios of A0A140G1P2; (B) the ratios of A0A140G1R2; (C) the ratios of A0A1S4DCM1.

Figure 9

Column map of PRM quantitative protein differences among different samples. (A) OM vs IM; (B) OM vs WM; (C) WM vs IM. The proteins corresponding to the column on the upside of transverse axis 0 represent up-regulated proteins, the proteins corresponding to the column on the underside of transverse axis 0 represent down-regulated proteins, numbers on columns represent multiple differences (non-logarithmic), for example: If the number in the underside column is 2, it means that WM is twice as much as IM in expression level; if the number in the upside column is 2, it means that WM is 0.5 times as much as IM in expression level.

Figure 10

External morphology of tobacco leaves with different maturity. (A), (D) IM; (B), (E) WM; (C), (F) OM.

Figure 11

Flow chart of protein separation and identification

Supplementary Files

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- [TableS2.xlsx](#)
- [TableS1.xlsx](#)
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