



## Proteome analysis of tobacco leaves reveals dynamic changes in protein expression among different cultivation areas

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**ABSTRACT.** The leaves of tobacco plants were used to analyze differences in protein content of tobacco grown in the four main flue-cured tobacco-producing areas of Sichuan Province, China. An improved protein extraction method, isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis two-dimensional gel electrophoretic separation, was used to extract and separate total protein from tobacco leaves. Proteomic maps with relatively high resolution and repeatability were produced. At isoelectric points 4 to 7 and molecular weight ranging from 20-100 kDa, we detected 1032, 1030, 1019, and 1011 clearly visible protein spots in tobacco leaves from the four study areas. Proteome comparison between these protein spots showed that 119 spots with a greater than 2-fold change in expression quantity contributed to the variation in expression. Of which, 115 were successfully identified and annotated. According to the annotation results, these proteins participate in photosynthesis, energy metabolism, mineral nutrition, terpene metabolism, defensive reaction, and other physiological and biochemical processes. This study preliminarily explains the effects of ecological conditions on

the physiological metabolism of tobacco leaves and how such effects directly or indirectly contribute to tobacco leaf quality.

**Key words:** Flue-cured tobacco; Proteome; Ecology; Leaf quality; Two-dimensional electrophoresis

## INTRODUCTION

The tobacco plant is an important commercial crop; however, its quality varies across its cultivation range. The difference in eco-climates between cultivation locations is a fundamental factor that results in great differences in tobacco leaf quality (Li and Dai, 2013). Many studies have been conducted to explore the mechanisms by which eco-climate affects tobacco quality, and the results have shown that some tobacco leaf quality indicators, such as dry matter accumulation, enzymatic activity, aroma substance content, sugar content, and other conventional chemical components of tobacco leaves, are significantly affected by either light wave length or by light intensity (Yang et al., 2007; Huang et al., 2009; Yun et al., 2010; Shi et al., 1999, 2012). Recently, plant proteomic analysis was used as a key tool to unveil mechanisms of tobacco leaf quality formation, and the results showed that differently expressed proteins were involved in plastid development, pigment metabolism, photosynthesis, glycolysis, photorespiration, defensive reaction, mineral nutrition, etc. (Cui et al., 2008; Chen et al., 2012b; Yang et al., 2012; Cai et al., 2013). These results indicate that differences in tobacco quality are caused by the differential expression of proteins involved in tobacco primary metabolism. However, these studies provide inadequate information about the proteins involved in other metabolic processes, such as secondary metabolism. Thus, we analyzed protein expression profiling of tobacco leaves from four ecological zones in Sichuan Province, China, which is an important high-quality tobacco production area, and our results show that global changes in tobacco leaf protein expression exist across the four areas.

## MATERIAL AND METHODS

### Plant materials

The flue-cured tobacco variety “Yunyan 87” was planted in Jiange, Xingwen, Miyi, and Huili in Sichuan Province, China. Tobacco seeds were sown on January 18, 2012, and seedlings were transplanted on May 1, 2012. Local conventional cultivation measures were used for field management. At 70 days after transplanting, the middle leaves of the 12th leaf position were obtained from 30 randomly selected plants and frozen in liquid nitrogen until required. At 120 days after transplanting, the middle leaves of the 12th leaf position from the remaining plants were collected and flue-cured according to the local tobacco production method.

### Conventional chemical components analysis of flue-cured tobacco leaves

Total soluble sugar (TSS), total nitrogen (TN), nicotine, potassium (K), chlorine (Cl), and starch content of the flue-cured tobacco leaves was measured according to Wang (2003).

## Protein extraction and two-dimensional electrophoresis (2-DE)

Tobacco leaf protein extraction and quantification were performed according to Wang et al. (2012) and Bradford (1976).

Before 2-DE was undertaken, isoelectric focusing was performed and finished in a GE isoelectric focusing spectrometer. The strips were pH 4.0-7.0, 17 cm IPG (Bio-Rad), and the loading quantity of sample proteins was 600 µg. The flow of the isoelectric focusing was as follows: 250 V fast boosting for 3 h, 200 V fast boosting for 2 h, 1000 V fast boosting for 1 h, and 10,000 V linear boosting for 3 h; focusing temperature was established at 20°C, and 10,000 V was maintained for 8 h; the limited current was set to 50 µA, and the product of running voltage x time reached 130,000 V/h. Then, the strips were equilibrated twice (approximately 15 min each time) using 1% DTT strip equilibrium liquid (Wang et al., 2012).

Next, the equilibrated strips were transferred to the upper side of a well-prepared SDS-PAGE (second dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with the glue facing outwards. The strip was completely exposed to the glue face. Subsequently, 1% low melting-point agarose with bromophenol blue was used for the enclosing step. After condensation of the agarose, the glue slab was placed into the Hoefer SE900 electrophoresis apparatus for the second dimensional vertical electrophoresis. The electrophoresis was in constant power mode. For pre-electrophoresis, 6 W was used for a single strip, and the electrophoresis lasted for 1 h; 8 W was adopted for each strip, and constant-power electrophoresis was adopted for 6 h.

## Gel staining and image scanning

After electrophoresis, the gels were stained according to Wang et al. (2012). An ImageScanner III (GE Healthcare) was used to scan the well-stained gel and to obtain images. The ImageMaster 2D Platinum (Amersham Bioscience) software was used to detect, match, and statistically analyze protein spots on the gel images. Three biological replications were selected for comparative analysis. Protein spots showing differences that reached the significance level ( $P < 0.05$ ) were obtained as differential protein spots. Protein spots with over 2-fold differential expression were used for mass spectrometry (MS).

## In-gel digestion of protein spots and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS

The targeted protein spots were removed and digested in the gel. Post-enzymolysis protein sample peptide fragments were sent to BGI Proteome Science Research Center (<http://www.genomics.org.cn>) for MALDI-TOF MS to produce the peptide mass fingerprinting map. The acquired sample mass spectrum data were analyzed using the MASCOT Distiller software.

## Bioinformatics analysis

The search engine of the matrix science website (<http://www.matrixscience.com>) was used to search and match data. The amino acid sequences of the identified proteins (with the highest MASCOT scores) were submitted to the COG database of NCBI (<http://www.ncbi>.

nlm.nih.gov/COG/old/xognitor.html) for protein COG functional analysis.

## RESULTS

### Conventional chemical component content analysis

We measured the tobacco quality indicators of TSS, TN, nicotine, K, Cl, and starch in flue-cured tobacco leaves from the four study areas. The results demonstrated that the content of these indicators in tobacco leaves varied across the four areas (Table 1). TSS content in tobacco leaves from Huili and Miyi was higher than the content in tobacco leaves from the other two study sites. In contrast, the starch content of tobacco leaves from Huili and Miyi was lower than the starch content in tobacco leaves from Xingwen and Jiange. However, no trends were observed for TN, nicotine, K, and Cl content in relation to the cultivation zones.

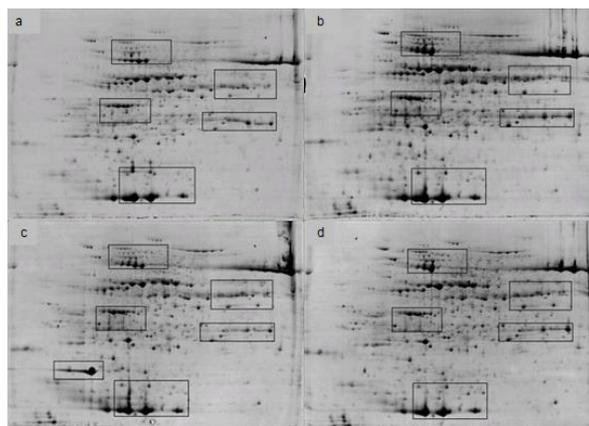
**Table 1.** Conventional chemical component content of flue-cured tobacco leaves from four different tobacco cultivation zones in Sichuan Province, China.

	TSS (%)	TN (%)	Nicotine (%)	K (%)	Chlorine (%)	Starch (%)
Huili	40.57 ± 0.617	1.96 ± 0.096	2.44 ± 0.061	1.82 ± 0.083	0.13 ± 0.006	4.84 ± 0.434
Miyi	41.10 ± 0.52	1.50 ± 0.034	1.38 ± 0.028	1.98 ± 0.038	0.07 ± 0.014	4.86 ± 0.473
Xingwen	28.70 ± 0.41	2.37 ± 0.053	3.36 ± 0.067	1.98 ± 0.064	0.14 ± 0.057	5.43 ± 0.382
Jiange	34.25 ± 0.427	1.96 ± 0.052	2.83 ± 0.112	1.59 ± 0.043	0.16 ± 0.024	5.26 ± 0.612

TSS = total soluble sugar; TN = total nitrogen; K = potassium. Data in the table are reported as means ± SE.

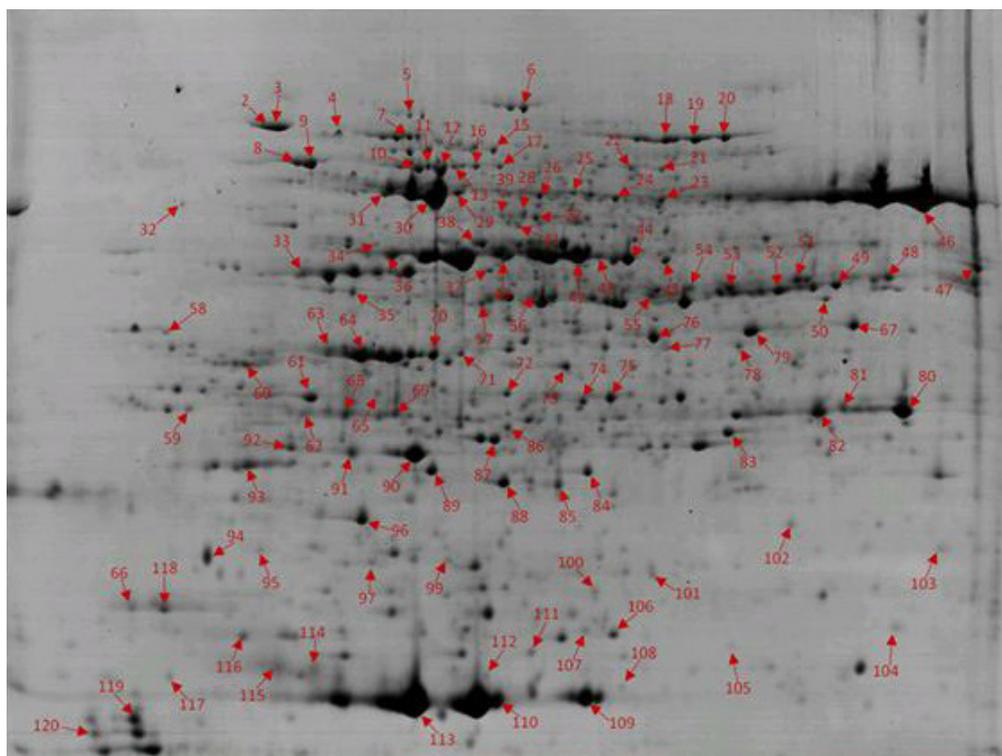
### 2-DE and 2-DE diagram analysis

The 2-DE technique was adopted to separate the total proteins of tobacco leaves. The gel background of the produced 2-DE diagrams was clean and clear, and most of the protein spot images were round with clear boundaries without trailing (Figure 1).



**Figure 1.** Two-dimensional gel electrophoretic separation of proteins from tobacco leaves grown in Jiange (a), Huili (b), Miyi (c), and Xingwen (d), Sichuan Province, China. The boxed areas show intensive areas of differently expressed protein spots.

The ImageMaster 2D Platinum software was used to analyze the electrophoretograms. Quantitative analysis results showed that 1032, 1030, 1019 and 1011 clearly visible protein spots were measured from the tobacco leaves sampled in Xingwen, Jiange, Huili, and Miyi, respectively. The protein spots were mainly distributed in the pH range 4.5-6.5 with molecular weights in the range 20-65 kDa. By using the protein abundance change of greater than 2-fold as a selection criterion, a total of 119 differentially expressed protein spots were obtained (Figure 2).



**Figure 2.** Identification of differentially expressed proteins of tobacco leaves grown in four regions of Sichuan Province, China, following two-dimensional gel electrophoretic separation. Red arrows represent differentially expressed proteins.

### Differentially expressed protein spots identified using MALDI-TOF MS

The resulting 119 differentially expressed protein spots were subjected to spot removal, in-gel digestion, MALDI-TOF MS, and MALDI-TOF/TOF MS. A total of 115 proteins were successfully identified by analyzing and matching the data obtained to data collected from database searches. The result also showed that several protein spots were classified as one protein, which may be caused by protein degradation, posttranslational modification, or the presence of multiple families (Song et al., 2007). The MS identification information and functions of some important proteins are classified in Table 2.

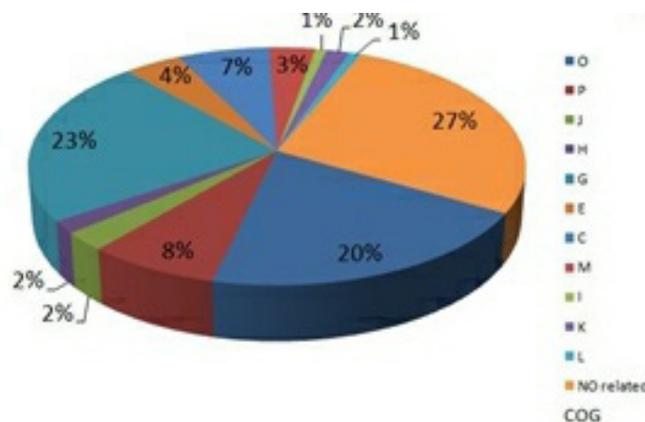
**Table 2.** Important proteins with known functions among the identified proteins from tobacco leaves grown in four different tobacco cultivation zones in Sichuan Province, China.

Spot No.	Accession No.	Protein identification and functional category	pI/Mr (kDa) The.	pI/Mr (kDa) Exp.	SC (%)
75, 80, 81, 82	gi 115473	Carbonic anhydrase, chloroplastic	6.41/34.9	5.47/35	36
27, 39	gi 157042757	Glucose-1-phosphate adenylyltransferase	6.19/57.5	5.19/53	18
48, 51	gi 120661	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	6.60/42.1	6.38/38	28
37, 42	gi 121373	Glutamine synthetase	5.51/39.0	5.16/41	15
47	gi 304368145	Glycolate oxidase	9.02/40.6	6.61/40	35
41	gi 157042741	1-hydroxy-2-methyl -2- (E)-butenyl 4-diphosphate reductase	5.60/52.0	5.24/51	12
61, 62, 65 67, 68, 69	gi 357512271	Chlorophyll a-b binding protein 21, chloroplastic	5.45/30.0	4.83/35	7
115, 116	gi 223593	carboxylase/oxygenase, RBP	4.99/14.7	4.65/15	34
119, 120	gi 130283	Plastocyanin	4.29/10.4	4.32/11	42

pI/mr The. = theoretical mass (kDa) and isoelectric point (pI) of proteins identified; pI/mr Exp. = experimental mass (kDa) and pI of proteins identified; Sequence coverage (SC, %), the amino acid SC for the proteins identified with peptide fragmentation fingerprint data.

### COG functional categories of differentially expressed protein spots

COG function search results for the differentially expressed proteins showed that the 115 proteins can be classified into 12 categories according to their biological functions (Figure 3). The dominant categories comprised proteins related to carbohydrate transportation, protein turnover, posttranscriptional modification, and molecular chaperones. Inorganic ion transportation, energy generation, and amino acid transportation were less dominant categories. However, a large proportion of proteins identified (27%) did not fall into any of the categories mentioned above (Figure 3, no related COG). Among the proteins identified, some proteins belonged only to one biological function category and others (e.g., protein spots 28, 41, 49, 72, and 73) showed multiple biological functions.



**Figure 3.** Functional classification of differentially expressed proteins from tobacco leaves grown in four regions of Sichuan Province, China. O, posttranslational modification, protein turnover, and chaperones; P, inorganic ion transport and metabolism; J, translation, ribosomal structure, and biogenesis; H, coenzyme metabolism; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; C, energy production and conversion; M, cell envelope biogenesis and outer membrane; I, lipid metabolism; K, transcription; L, DNA replication, recombination, and repair; No related COG, protein COG function does not belong to any class.

## DISCUSSION

In our study, the proteomes of tobacco leaves from different ecological zones in Sichuan Province were analyzed and compared. A total of 119 differentially expressed protein spots were identified, among which, 115 protein spots were successfully annotated using MS identification. These proteins are mainly involved in photosynthesis, carbohydrate transportation and metabolism, protein turnover, posttranscriptional modification, and molecular chaperone.

### **Chlorophyll a/b-binding proteins and plastocyanin**

Light enhances the growth and development of plants by signals and energy. Photo-reaction is the first and vital reaction of photosynthesis. PSII chlorophyll a/b-binding proteins (protein spots 61, 62, 65, 67, 68, and 69) and plastocyanins (protein spots 119 and 120) are important components of photosynthesis (Jansson, 1994; Li, 2006). The results of previous research demonstrated that lower light intensity resulted in lower TSS content (Yang et al., 2007). In our study, proteins identified as participating in photosynthesis indicate the presence of variations in terms of light energy utilization and conversion in tobacco plants grown in different regions. Huili and Miyi are areas with high intensities of sunlight and extremely abundant photo-thermal resources. Thus, our study helps to explain the results of Yang et al. (2007), where lower light intensity resulted in lower TSS content, and underlies the higher TSS content of tobacco leaves from the Huili and Miyi regions in comparison to the other areas.

### **Glyceraldehyde-3-phosphate dehydrogenase and glucose-1-phosphate adenylyltransferase**

Glyceraldehyde-3-phosphate dehydrogenase (protein spots 48 and 51) catalyzes the conversion of 3-phosphoglycic acid into glyceraldehyde-3-phosphate in the Calvin cycle, completing carbon fixation and depositing energy (Li, 2006). The enzyme glucose-1-phosphate adenylyltransferase (protein spots 27 and 39) is crucial for starch synthesis (Chen et al., 2012a). The differential expressions of the above-mentioned enzymes indicate that differences exist in the dark reactions during photosynthesis and in starch synthesis in tobacco from the four ecological zones of Sichuan Province. Thus, these differences will lead to differences in the content of carbohydrate products, such as TSS and starch, in tobacco leaves from the four regions.

### **Glycolate oxidase, carbonic anhydrase, and Rubisco**

Glycolate oxidase (protein spot 47), carbonic anhydrase (protein spots 75, 80, 81, and 82), and Rubisco (protein spots 75, 80, 81, and 82) are enzymes that are significantly correlated with the rate of plant photosynthesis (Zelitch, 1966; Spreitzer, 2003; Wu et al., 2006; Mei et al., 2007). Thus, the differential expression of these enzymes in leaves of tobacco collected from the different ecological zones of Sichuan Province suggests that tobacco plants growing in these zones may differ in their rates of photosynthesis. The differences in photosynthetic rate may result in differences in development and quality of tobacco plants from these different areas.

## Glutamine synthetase (GS)

GS (protein spots 37 and 42) participates in nitrogen metabolism, is the key enzyme that regulates the assimilation of inorganic nitrogen into organic nitrogen, and it catalyzes the generation of glutamine by ammonium ions and glutamic acid. The activity of GS may be related to illumination intensity, light quality, temperature, and day-night temperature gap (Yu et al., 2012). Thus, light and temperature differences in different tobacco cultivation zones may lead to different expression levels and activity of GS, and thereby result in various levels of nitrogen metabolism, eventually affecting tobacco leaf quality.

## 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR)

We also identified the protein HDR (protein spot 41), which participates in plant terpene substance synthesis, and is the rate-limiting enzyme for the synthesis of terpene compounds (Zhang et al., 2008). Terpene compounds are important secondary metabolites of tobacco plants, of which monoterpene, sesquiterpenes, and diterpenoids are the main components of tobacco blade surface excretions and are closely associated with plant resistance and the aroma qualities of tobacco leaves. The other terpene compounds, such as carotenoids, solanesol, and labdanoids, are the main aromatic substances or precursor substances of tobacco plants and contribute to tobacco leaf quality (Wanger, 1999). The differences in HDR expression quantity among the tobacco planting zones may have resulted in differences in terpene substances in the tobacco leaves, and consequently, a difference in tobacco leaf quality, among the zones.

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