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Metabolic profiling of tobacco leaves at different growth stages or different stalk positions by gas chromatography–mass spectrometry

Li Zhang[a], Xiaotao Zhang[a], Houwei Ji[a], Weiwei Wang[a], Jian Liu[a], Fang Wang[a], Fuwei Xie[b], Yongjie Yu[c], Yaqiong Qin[b], Xiaoyu Wang[b,*]

[a] Technology Center, China Tobacco Gui Zhou Industrial Co., Ltd., Guiyang 550009, PR China
[b] Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou 450001, PR China
[c] Ningxia Medical University, Yinchuan 750004, PR China

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ABSTRACT

The dynamic and temporal changes in tobacco leaf metabolites can have an immense impact on their compositions, which are closely related to the quality and flavor of the tobacco leaves. To investigate the dynamic changes in metabolites during tobacco growth and development and the diversity of metabolites in tobacco leaves from different stalk positions, a gas chromatography–mass spectrometry (GC–MS) method with derivatization was developed to profile tobacco leaves. A hierarchical cluster analysis (HCA) enabled the assignment of five types of tobacco leaves (the middle leaves in the resettling, vigorous, maturity stage and lower, and upper leaves in the maturity stage) to different groups. The differences among the samples at different growth stages are much more obvious than those from different sampling positions. Using the partial least squares-discriminant analysis (PLS-DA) model, the metabolites with distinct differences were screened out. During tobacco development, the contents of the metabolites related to sugar metabolism, amino acid metabolism, shikimic acid metabolism and terpenoid metabolism all increased at first and then decreased, while the contents of the tricarboxylic acid cycle intermediates, such as citric acid and fumaric acid, both decreased at first and then increased. The alkaloid contents increased significantly throughout the developmental period, and the metabolite contents related to organic acid decreased significantly during the maturity stage. Among the tobacco leaves from different stalk positions in the maturity stage, the sugar contents were highest in the middle leaves, while the organic acid contents were highest in the lower leaves. In addition, the nicotine, chlorogenic acid and α-4,8,13-duvatriene-1,3-diol contents were highest in the upper leaves. Apart from insights into the metabolite changes that occurred during tobacco leaf development, these results also provide potentially valuable information for regulating the primary metabolites in the future.

1. Introduction

Tobacco (*Nicotiana tabacum* L.), a dicotyledonous *Solanaceae* plant, is not only an important industrial crop and the primary raw material of tobacco commodities, but it is also an important model plant for studying molecular biology and genetic engineering. Tobacco plants underwent a series of physiological and biochemical metabolic processes during the growth and development period. The dynamic and temporal changes in tobacco leaf metabolites can have an immense impact on their compositions, which are closely related to the quality and flavor of the tobacco leaves (Thielen et al., 2008). In addition to temporal regulation, the chemical compositions of tobacco leaf largely depend on the environment, genetics and spatial position. Therefore, investigating the contribution of these multi-dimensional regulations to the broad diversity of metabolites will provide new insights into the biochemical pathways and the formation mechanisms for significant flavor components and undesired harmful compounds. Studies on the effects of the development, environment, genetics and spatial position have been widely reported. However, only several conventional chemical components in tobacco, such as phenolic compounds and phytosterols, were studied in those reports (Andersen et al., 1972; Liu et al., 2008), which were not comprehensive and systematic. A comprehensive and systematic investigation of chemical components based on metabolomics would broaden the understanding of the relationship between leaf metabolism and these multi-dimensional regulations.

Metabolomics, which aims at the comprehensive analysis of metabolites, is playing an important role in the study of the dynamic metabolic process to reveal the essence of life's activities (Musilova and

* Corresponding author.
E-mail address: wangxiaoyu@iccas.ac.cn (X. Wang).

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Glatz, 2011; Tang and Wang, 2006). Since Sauter et al. (1991) first introduced the concept of metabolic profiling in the plant system in the early 1990s, metabolic profiling has been widely used in *Arabidopsis thaliana*, rice, medicinal plants and algae, etc. (Fiehn et al., 2000; Fiehn, 2002, 2003; Duan et al., 2012; Roessner et al., 2000; Wei et al., 2013; Tarpley et al., 2005). Recently, the metabolic profiling method has also been applied to research on tobacco leaves (Ma et al., 2012; Li et al., 2011; Zhang et al., 2011b; Zhao et al., 2013, 2014, 2015, 2016). Zhang et al. (2013) systematically investigated the metabolic profiling of tobacco leaves from different geographical origins and screened out some important metabolites related to the planting regions and climate factors. These results also indicated that the planting environment has a greater effect on the metabolic changes than the genetics. However, there are few reports on the metabolic profiles of tobacco leaves at different stalk positions or at different growth stages. In this paper, we will focus on the dynamic changes in metabolites during tobacco growth and development, and the diversity of metabolites in tobacco leaves from different stalk positions.

At present, the analytical methods used in the metabolic profiling study primarily include gas chromatography mass spectrometry (GC–MS) (Zhao et al., 2015, 2016), liquid chromatography tandem mass spectrometry (LC–MS) (Chang et al., 2012; Li et al., 2014), capillary electrophoresis–mass spectrometry (CE–MS) (Ramautar et al., 2009; Zhao et al., 2014), and nuclear magnetic resonance (NMR) (Kim et al., 2011). Compared with LC–MS, CE–MS or NMR, GC–MS has the advantages of good repeatability, commonly used mass spectrum databases, and the ability to analyze a wide range of metabolites, including alcohols, phenols, amines, terpenoids, terpene alcohols, amino acids, and fatty acids by derivatization (Lisec et al., 2006; Tsugawa et al., 2011). A pseudo-targeted metabolic profiling method based on GC–MS with a selective ion monitoring mode has been developed and applied for the discrimination of tobacco leaves from different geographical origins, and the sensitivity, accuracy and precision are improved by using this method compared with the common full scan (SCAN) mode (Li et al., 2012). In this study, a pseudo-targeted metabolic profiling method based on GC–MS will be used to investigate the influence of the growth stages and stalk positions on the metabolites in tobacco leaves.

2. Materials and methods

2.1. Materials

Five samples at different growth stages or from different stalk positions (flue-cured type) were collected from Guizhou province, in southwestern China. Six collections taken from an individual plant that grew in the same field were obtained for each sample (Table 1). In total, thirty collections were used for the tobacco metabolic profiling analyses. The newly collected green tobacco leaves were immediately frozen in liquid nitrogen and stored at −80 °C. Prior to analysis, these green tobacco leaves were freeze-dried for 24 h and then ground to powder and filtered through a 40-mesh sieve (Gullberg et al., 2004; Kim and Verpoorte, 2010). The tobacco powder was stored at −80 °C. A quality control (QC) sample was obtained by blending the same amount of each milled collection thoroughly. During this study, the QC sample was primarily used to establish the SIM method and to monitor the quality of the entire analysis.

Table 1
Collected samples of tobacco leaves at different growth stages or different stalk positions.

Index	Growth stage	Sampling position	Sampling date	Sampling location	Species
A	Rosette Stage (R)	Middle(M)	16 June	Guizhou province ZunYi city	K326
B	Vigorous Growth Stage (V)	Middle(M)	6 July		
C	Maturing Stage(M)	Lower(L)	28 July		
D		Middle(M)	28 July		
E		Upper(U)	18 August		

2.2. Reagents

All the solvents used in the study were HPLC grade. Methanol and chloroform were purchased from J. T. Baker (PA, USA). Forty standard substances with purity levels higher than 98%, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), methoxyamine hydrochloride and pyridine were all purchased from J&K (Beijing, China). Hexadecanoic-7,7,8,8-d_4 acid was purchased from CDNISOTOPESInc (Quebec, Canada). Distilled water was purified "in-house" using a Milli-Q system by Millipore (MA, USA).

2.3. Sample preparation

The green tobacco metabolites were extracted with a single-phase solvent mixture of MeOH, H_2O, and $CHCl_3$ at a ratio of 5/2/2 (v/v/v) as previously reported (Zhang et al., 2013). In brief, twenty milligrams of green tobacco powder was weighed and transferred into a 5-mL Eppendorf tube, and then 2 mL of solvent mixture and 200 μL of 40 μg/mL hexadecanoic-7,7,8,8-d_4 acid were added sequentially. The mixture was sonicated for 40 min and centrifuged at 10,000*g* for 10 min (SIGMA 3k15, Germany). Then, 400 μL of the supernatant was completely dried with pressurized gas blowing concentrators (Organomation N-EVAP, USA). Oximation and silylation were then performed sequentially. First, fifty microliter of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to the dried residue. Then, the mixture was vortexed for 1 min and then oximated at 37 °C for 90 min. Finally, the oximated sample was silylated with 70 μL of *N*-methyl-*N*-(trimethylsilyl) −trifluoroacetamide (MSTFA) at 37 °C for 30 min. After standing at room temperature for at least 1 h, the derivatized samples were injected into the GC/MS.

2.4. GC/MS analysis

The samples were analyzed in a random order, with QC samples being inserted at every eight samples into the sequence as previously described (Zhang et al., 2013). The samples were analyzed using an Agilent 7890-5975 GC/MS system equipped with an Agilent G4513A auto-sampler (Agilent, Atlanta; GA). One microliter of the TMS-derivatized sample was separated by a DB-5 MS fused-silica capillary column with a 30 m length, 0.25 mm inner diameter and 0.25 μm film thickness (Agilent Technologies, Palo Alto, CA) with a split ratio of 5:1. The inlet temperature was 290 °C and the flow rate of high-purity helium gas (≥99.999%, China), which was employed as the carrier gas through the column, was 1 mL/min. The initial oven temperature was held at 70 °C for 4 min, and it was then ramped up at 5 °C/min to 310 °C for 10 min. The transfer line and the ion source temperatures were 280 °C and 230 °C, respectively. The ionization mode was the electron impact (EI) and the EI voltage was 70 eV. A mass scan range of 40–510 *m/z* was used for full scan mode at an acquisition rate of five scans per second. The acceleration voltage was turned on after a solvent delay of 8 min. A light diesel oil sample (C_{10}–C_{30}) was analyzed in full scan mode to calculate the Kovat's retention index (RI) of the detected metabolites.

2.5. Data preprocessing

The acquired GC–MS raw data from the QC sample was analyzed with the Automatic Mass Spectral Deconvolution and Identification System (AMDIS) (NIST, Gaithersburg, MD) to verify the presence of individual analytes and to deconvolute the co-eluting peaks as