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Full Length Research Paper

Quantitative determination of DNA Methylation in tobacco leaves by HPLC

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A relatively straightforward high performance liquid chromatography (HPLC) method for determination of DNA methylation in flue-cured tobacco leaves was developed and validated. DNA methylation was measured as follows: DNA was hydrolyzed to nucleotide units in perchloric acid. The bases of cytosine and 5-methylcytosine were separated on a reversed-phase C18 column at 30°. The mobile phase used methanol: 5 mmol/L pentane sulfonate (10:90, v/v) and a flow rate of 0.7 mL/min. The absorbance of bases was measured at 273 nm. The results showed that the method was accurate, quick, and cost effective to determine DNA methylation of flue-cured tobacco.

Key words: Tobacco, DNA methylation, 5- Methylcytosine, high performance liquid chromatography (HPLC).

INTRODUCTION

DNA methylation in plants plays an essential role in plant gene expression (Razin and Cedar, 1991; Kuroda et al., 2009), development (Burn et al., 1993), aging (Demeulemeester et al., 1999), and the epigenetic signal (Ginder et al., 2008) to suit the changes of environments. Although nucleotides bearing the major purines and pyrimidines are most common, the nucleotides of DNA in different plants could not be separated by the same method; as the mobile phase, flow rate, column temperature, and components in plants would affect the separation of cytosine and 5-methylcytosine. Tobacco (*Nicotiana tabacum* L.) is one of the economic crops which is sensitive to the environments, and used as a model plant to study the development and metabolism of plants (Masclaux et al., 2000; 2002). Moreover, tobacco contains a large number of primary and secondary metabolites, particularly the mature leaf that contains about a thousand-kind of chemical substances, which makes it difficult for the separation and determination of the level of methylation. Therefore, it is indispensable to build a method for researching the relationships between tobacco's DNA methylation and its development and metabolism.

High performance liquid chromatography (HPLC) is the most commonly used chromatographic method for analysis of the genome-wide methylation from Wagner and Capesius (Wagner and Capesius, 1981). The universal HPLC methods provide absolute and quantitative information, however these methods were time-consuming and expensive. Firstly, many universal methods were relatively slow to detect DNA methylation from 20 to 80 min with HPLC. Secondly, DNA was hydrolyzed by enzymes for about 24 h to prepare samples (Schiewek et al., 2007; Sandhu et al., 2009). Thirdly, other methods to detect DNA methylation need especial detection (Li et al., 2009), and the complex program to elute the column with gradient mobile phase (Sandhu et al., 2009). Moreover, the column temperature was higher (Demeulemeester et al., 1999). In response to research the relationships between DNA methylation and development or quality of tobacco, we have developed a relatively straightforward and cost effective HPLC method compared with the formers for determination of DNA methylation in tobacco.

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MATERIALS AND METHODS

Instrument and reagents

In this study, analysis was performed with a Dionex HPLC system (P680 LPG pump, UVD170U detector, TCC-100 oven, and Rheodyne7725i manual injector) controlled by Chromeleon chromatographic data station (Dionex, USA). Separation was on a Nucleosil C18 column (250 × 4.6 mm, 5 µm). The detection wavelength was 273 nm. The injection volume was 20 µL. Cytosine and 5-methylcytosine standards were obtained from Alfa Aesar (A Johnson Matthey Company, USA). Pentane sulfonate (PICB5) (HPLC grade) was obtained from Yuwang (Yuwang, China). Methanol (HPLC grade) was obtained from Sayfo (Sayfo, China). Water was purified with an ultra-pure water system (Milliopre, SAS, France). The other reagents were obtained from Beijing Chemical Co., Ltd (China).

Preparation of standard solutions and mobile phases

The standard stock solutions were prepared by 10 mg of 5-methylcytosine and cytosine dissolved in 50 ml ultra-pure water, respectively. The standard working solutions were diluted by the standard stock solutions with stepwise method to gain the different concentrations of 50.0, 25.0, 10.0, 5.0, 1.0 mg/L. Three mobile phases were prepared as follows: mobile phase I, methanol: pentane sulfonate (5 mmol/L) (10:90, v/v), mobile phase II, methanol: pentane sulfonate (10 mmol/L) (10:90, v/v), and mobile phase III, 0.05 mmol/L KH_2PO_4, respectively. The resulting solutions were filtered through a 0.45 µm Millipore filter into a vial before injection.

Validation of the method

The method was validated for linearity, accuracy, and precision. Linearity was determined with a series of cytosine and 5-methylcytosine standard solutions at five different concentration levels. The equation of the calibration plot was determined by unweighted linear regression. Accuracy was expressed as recovery ratio and evaluated in terms of repeatability at standard added level (0.1 µg) using six replicates for the same sample with cytosine or 5-methylcytosine standard solutions, respectively. The precision of the method was evaluated at concentration (10.0 mg/L) of standard solution and a sample using six replicates each. The LOD was defined for a S/N of 3.

Preparation sample

Genomics DNAs were isolated from tobacco leaves with the CTAB method. The method of DNA hydrolysis was determined according to Demeulemeester et al. (1999). The contents of cytosine and 5-methylcytosine calculated with external standard method. The global DNA methylation level = peak area of 5-methylcytosine / (peak areas of cytosine and 5-methylcytosine) × 100%.

RESULTS AND DISCUSSIONS

Effects of mobile phases on separating cytosine and 5-methylcytosine

The mobile phase I and II were able to separate cytosine and 5-methylcytosine effectively. However, the mobile phase III could not separate cytosine and 5-methylcytosine availability; the most likely reason for this is that some materials from DNA extraction affect the separation of Cytosine and 5-methylcytosine with KH_2PO_4 as mobile phase.

Effects of column temperatures and flow rates on separating cytosine and 5-methylcytosine

The cytosine and 5-methylcytosine could separate effectively when the column temperatures selected at 25, 30, 35, and 40℃, respectively. Because the higher the column temperature is, the shorter the life span of column is. The column temperature was chosen at 30° for stability of test conditions. The flow rates were selection of 0.5, 0.6, 0.7, and 0.8 mL/min, respectively. The column pressure was higher when the flow rate was faster. When the flow rate was greater than 0.7 mL/min, the pressure of the system was excessive large. The system of chromatography was suitable when the flow rate selected at 0.7 mL/min.

Validation of the method

The results of HPLC with standard working solutions (1.0, 5.0, 10.0, 25.0, 50.0 mg/L) are presented in Table 1. The level of cytosine gave the higher recovery ratio of 102.18% with the lower relative standard deviation (RSD); the level of 5-methylcytosine gave the lower recovery and high RSD. The precisions for the cytosine and 5-methylcytosine were less than 0.11 and 0.13%, and the precisions of the sample were 3.80 and 4.28%. Limit of detection (LOD) of the method for mixed standards was 50 µg/L. The results showed that the regression equations had a good linearity between concentrations and peak areas, therefore, the precision and accuracy of assay were acceptable.

Separating cytosine and 5-methylcytosine of standards and determination of samples

Under the selected chromatographic conditions, column temperature at 30° using the mobile phase I and the flow rate at 0.7 mL/min, the cytosine and 5-methylcytosine in mixed standards and samples were able to separate in a shorter time. The peaks of cytosine and 5-methylcytosine were separated significantly from biological noise in the standards. The retention time of cytosine and 5-methylcytosine were about 6.901 and 8.310 min with a total run time of 15 min (Figure 1). Chromatographic resolution (Rs) in this separation is 3.12. The global DNA methylation levels, presented during leaf senescence, were detected under the choose chromatography (Figure 2). The level of DNA methylation was approximately 3-fold higher in old leaves (50 d) than in young leaves (10 d), and reached a maximum in old leaves. In short, this result

Table 1. Validation results of HPLC method for the determination of cytosine and 5-methylcytosine in tobacco leaves.

Parameter	Results
Regression equation	
Cytosine	Y=420.56X -280.73 (R2=0.9985)
5-methylcytosine	Y=408.71X -329.25 (R2=0.9993)
Linear range (mg/L)	
Cytosine	1.0-25.0
5-methylcytosine	1.0-25.0
Accuracy	
Mean ± RSD of cytosine (%)	102.18 ± 2.59
Mean ± RSD of 5-methylcytosine (%)	96.67 ± 3.31
Precision	
Mean ± RSD of cytosine (%)	0.1022 ± 0.11
Mean ± RSD of 5-methylcytosine (%)	0.0967 ± 0.13
Mean ± RSD of sample (%)a	0.339 ± 3.80
Mean ± RSD of sample (%)b	0.073 ± 4.28
LOD (μg/ L)c	50
RSD (%)d	3.71-9.68

a. Cytosine concentration of the sample, n = 6; b. 5-methylcytosine concentration of the sample, n = 6. c. Mixed standard of cytosine and 5-methylcytosine, n = 6; d. Six samples from three areas, n = 4 for each.

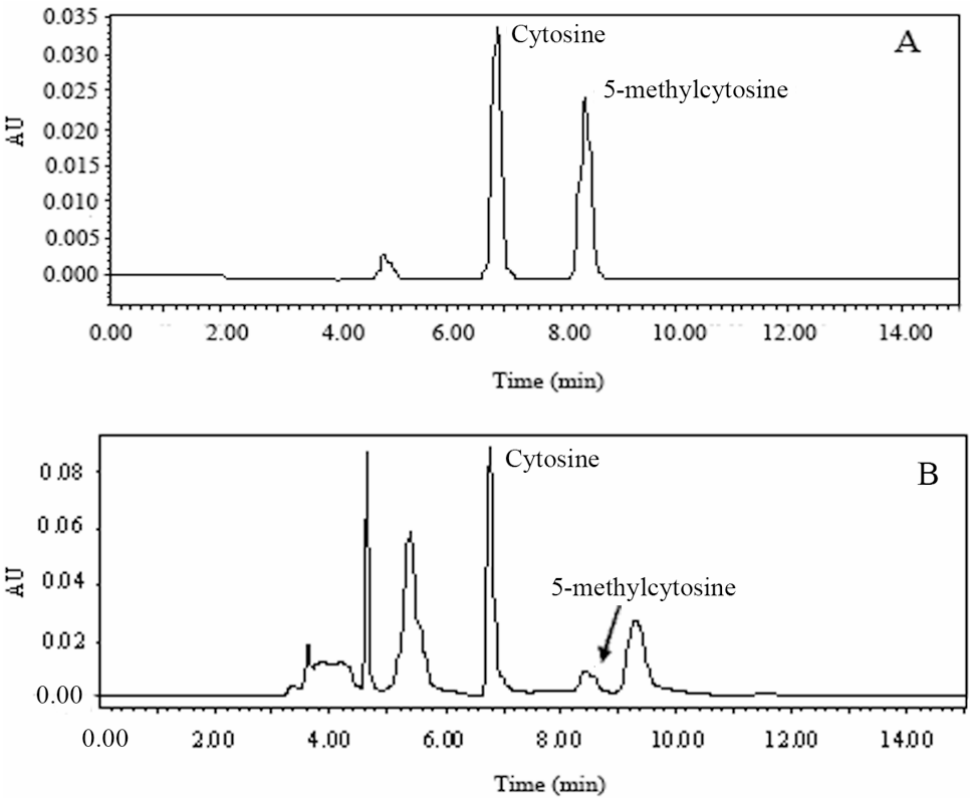

Figure 1. Chromatograms of standards (A) and sample of tobacco (B). The peaks marked are peaks of cytosine and 5-methylcytosine, respectively. The retention time of cytosine and 5-methylcytosine were about 6.901 and 8.310 min.

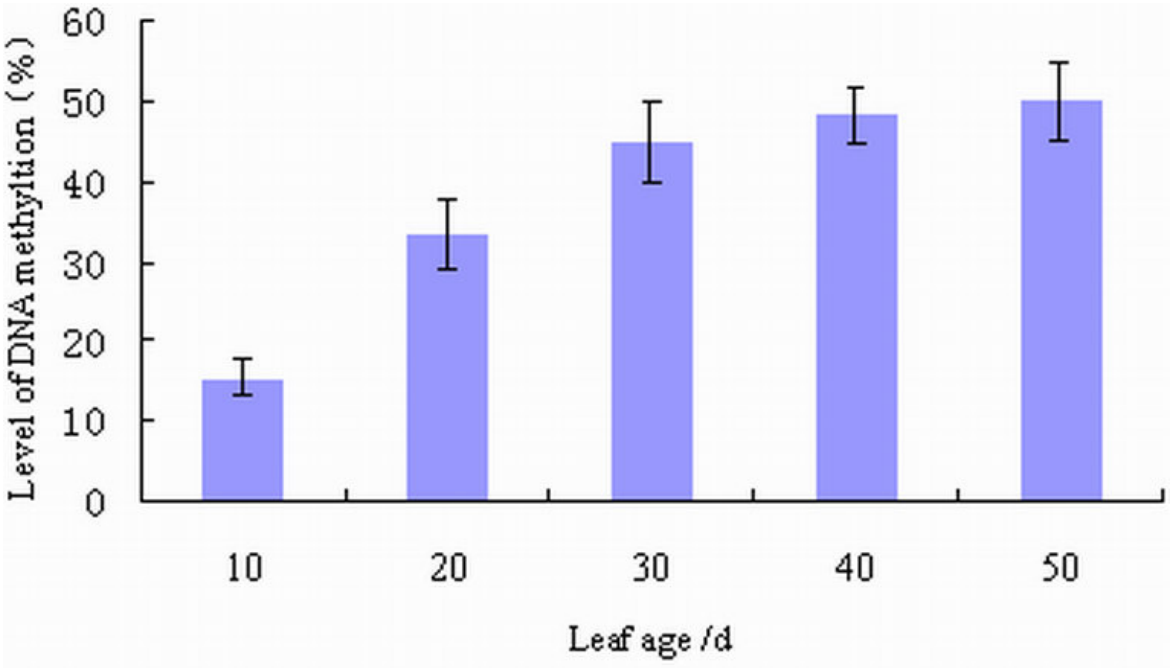

Figure 2. Level of DNA methylation ± S.E. (n = 5) of leaves during the flue-cured tobacco leaf aging process.

in accordance with the rule of tobacco aging and showed that the method to determinate DNA methylation of flue-cured tobacco was accurate, quick, and cost effective.

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