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Different tobacco cultivation regions lead to variations in tobacco leaf gene expression profiles involved in carbonhydrate metabolism and ion transportation

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Abstract

Tobacco is an important crop economically in China, and tobacco quality varies across its cultivated regions. To unveil the mechanisms that result in such varied tobacco leaf qualities, we have analyzed the tobacco leaf gene expression profiles of four areas in Sichuan province, China. Tobacco leaves in the 12th position were collected from plants in four different regions, namely Huili, Miyi, Xingwen and Jiange, and submitted for microarray analysis. Gene expression levels from Miyi group were used as control, and that from Jiange, Xingwen and Huili were compared to Miyi group respectively. A total of 5154 differentially expressed genes (DEGs) were detected, among which 2731 genes were up-regulated and 2423 genes were down-regulated. Based on gene ontology (GO) analysis, significant DEGs were classified into 15 categories ($P \leq 0.05$) based on properties pertaining to transferase activity, transmembrane transporter activity, binding, transcription regulator activity, metabolic processes, secondary metabolic processes, etc. Our results also show that these DEGs were significantly enriched in 11 pathways, including metabolic pathways, starch and sucrose metabolism, photosynthesis, etc. Our study suggests that many of the genes involved in various plant physiological and biochemical processes significantly differed across these four different regions, which may explain their differences in tobacco leaf quality.

Keywords: *Nicotiana tabacum* L, transcriptome, ecology, quality, gene chips.
Abbreviations: AGP_ADP glucose pyrophosphorylase; DEGs_differentially expressed genes; GER_ germin-like protein; GO_gene ontology; HTA_*Helanthus tuberosus* agglutinin; NCED_9-cis-epoxycarotenoid dioxygenase; qPCR_quantitative reverse transcription polymerase chain reaction; RIP_ribosome inactivating protein.

Introduction

Tobacco is an important commercial crop in China. Because tobacco with high leaf quality can be sold at higher prices, farmers are more concerned with tobacco leaf quality than with yield. Leaf quality varies across tobacco cultivation areas and can be determined based on certain conventional chemical indicators, such as total sugar, total nitrogen, nicotine, starch and potassium content, etc (Lu et al., 2015). Among the many factors that can affect tobacco leaf quality, eco-climate is particularly important (Li and Dai, 2013). Some studies (Shi et al., 1999; Yang et al., 2007; Huang et al., 2009; Yun et al., 2010; Shi et al., 2012) have revealed that light wave length and/or light intensity can significantly affect tobacco leaf dry-matter accumulation, aroma substance content, enzymatic activity, sugar content, etc. Although these individual studies unveil certain parts of the mechanisms related to the effects of eco-climate on tobacco leaf quality, the overall mechanism can only be understood by analyzing gene expression profiles across the entire genome. Recently, transcriptome analysis technology has been widely used to unveil the mechanisms involved in plant responses to abiotic stresses (Wasaki et al., 2003; Misson et al., 2005; Lian et al., 2006; Wasaki et al., 2006; Li et al., 2010; Krapp et al., 2011; Wang et al., 2011; Woo et al., 2012; Ma et al., 2012). This technology has also been used to investigate the mechanisms that determine different crop qualities. Long et al. (2011) have compared the gene expression profiles of flax obtained from the Hunan and Yunnan provinces in China and classified most of the differentially expressed genes detected under the functional category of cell structure.

In tobacco, gene expression profile analysis was also used to unveil the relationship between eco-climate and tobacco leaf aroma characteristics (Cui et al., 2011; Zhang et al., 2014). The result showed that differences in carbon metabolism, phenylpropanoid synthesis, alkaloid synthesis and terpenoid metabolism may account for the different tobacco leaf aroma characteristics of the Henan, Yunnan and Guizhou regions. However, tobacco leaf aroma characteristics is only one aspect of tobacco leaf quality. The relationship between conventional chemical components, which mainly determine tobacco leaf quality, and eco-climate at gene expression level remains unknown. Sichuan Province is an important tobacco cultivation region in China, and Miyi, Jiange, Xingwen and Huili are four typical tobacco cultivation areas in eco-climate in this province. Among them, tobacco leaf quality from Miyi is considered the best in the province. Lu et al. (2015) have measured the total sugar, total nitrogen, potassium and starch contents of leaves obtained from the four typical tobacco cultivation areas. The results showed that total sugar contents of leaves from Huili and Miyi were higher than those of the other two sites, while the starch contents of the former were lower than those from Xingwen and Jiange. Therefore, to uncover the relationship

between tobacco conventional chemical components formation and eco-climate at gene expression level we collected tobacco leaves from these four ecological zones, and analyzed their gene expression profiles. Our results revealed that global changes in tobacco leaf gene expression regarding photosynthesis and ion transportation existed across the four areas.

Results and Discussion

Tobacco gene expression profiles indicate global changes across tobacco cultivation areas

We analyzed tobacco leaf gene expression profiles in leaves cultivated in Xingwen, Jiange, Huili and Miyi during the tobacco topping period and compared the strengths of gene expression signals in leaves collected from these regions. We considered 2-fold or greater changes in gene expression ratios to indicate up-regulation and 0.5-fold or lower changes in gene expression ratios to indicate down-regulation. At topping period, compared to Miyi a total of 5154 differentially expressed genes were detected, among which 2731 genes were up regulated and 2423 were down regulated (Table 1). Among these 5154 DEGs, 359 genes were shared by tobacco leaves from the Xingwen, Jiange and Huili regions, including 192 up-regulated genes and 167 down-regulated genes.

To reveal the similarities and differences in genes expression profiles obtained for leaves collected from different tobacco cultivation regions, we performed a hierarchical cluster analysis. The results showed that genes expression patterns were similar among tobacco leaves from the Xingwen, Jiange and Huili regions and differed from the patterns observed in tobacco leaves obtained from the Miyi region (Fig. 1). To verify the microarray data obtained, we performed qPCR analysis of the 12 randomly selected genes and found that the expression patterns of 10 out of 12 genes agreed quite well with the corresponding microarray data (Fig. 2).

Functional classification of differentially expressed genes

We classified the functions of these DEGs by means of GO (Gene Ontology) analysis (http://www.geneontology.org) and found that these DEGs were significantly enriched with regard to 15 classifications ($P \leq 0.05$), including transferase activity, binding, etc. (Fig. 3). These 15 classifications included genes with up- or down- regulated expression levels. The percentages of down-regulated genes showing enrichment with regard to these 15 classifications were higher than those obtained for up-regulated genes, except in the case of transcriptional activator activity. These results suggest that the transcriptional activator activity of tobacco leaves cultivated in Miyi was lower than that of the other three regions.

Differential expression genes enriched metabolic pathway analysis

Differentially expressed genes were significantly enriched in 11 pathways (Table 2), among which only the metabolic pathway simultaneously existed in all three comparison groups. Pathways existing in two comparison groups at the same time were endocytosis, thiamine metabolism, starch and sucrose metabolism, and photosynthesis. Because starch and sugar are tobacco leaf quality indicators, differences in the expression patterns of associated genes may indicate that primary metabolism, such as photosynthesis and starch and sucrose metabolism, and secondary metabolism such as carotenoid and flavonoid biosyntheses play key roles in determining tobacco output and quality.

DEGs in photosynthesis

Light reaction is a process involving light collection and energy-conversion and is, therefore, vital for plant photosynthesis (de Bianchi et al., 2011). The present study has identified six differentially expressed genes (DEGs) involved in the light reaction of photosynthesis (Table 3), all of which encode proteins involved in photosystem I or photosystem II. *LHC* genes encode light-harvesting complex proteins; thus, *LHC* expression levels may change under conditions of drought stress and/or according to light strength (Peremarti et al., 2014; Chen et al., 2010). The observed down-regulation of all six of these DEGs may indicate more efficient light collection and energy conversion in tobacco leaves from the Miyi region compared to the other three areas, which may account for the higher sugar content measured in leaves collected from the Miyi region (Lu et al, 2015).

Carbon fixation is an important biochemical reaction that is essential for photosynthetic sugar synthesis. Upon comparing the gene expression profiles for tobacco leaves obtained from the Huili and Miyi regions, we have identified three DEGs involved in the carbon fixation process (Table 4). Of these three genes, *SBPase* was up regulated, and *PPC3* and *BCA3* were down regulated. *SBPase* is vital to the Calvin circle in photosynthesis. Previous studies have shown that, in transgenic organisms, overexpression of *FBP/SBPase* genes enhances CO_2 assimilation rates and increases biomass production (Miyagawa et al., 2001; Yabuta et al., 2008; Ichikawa et al., 2010; Ogawa et al., 2015). Higher expression of *SBPase* may explain why the sugar contents of tobacco leaves are higher in the Huili region compared to the Miyi region.

Because carbonic anhydrase (CA) plays a key role in the transformation of CO_2 to HCO_3^-, which can be utilized for carbon fixation, changes in *CA* gene expression levels and carbonic anhydrase activity may result in changes in plant photosynthesis and, eventually, differences in plant development (Sun et al., 2001; He et al., 2012). Therefore, our results suggest that changes in the expression levels of genes involved in tobacco photosynthesis may lead to differences in tobacco photosynthesis and sugar contents across cultivation areas.

DEGs in starch and sucrose metabolism pathway

Transcriptome profiling reveals differences in starch and sucrose metabolism across the four regions considered herein. Because sugar has an important physiological role in providing energy as well as the carbon frames in fresh tobacco leaves, it may also greatly influence the smoke quality of tobacco leaves. To some extent, tobacco leaf quality improves with increased sugar content (Wang et al., 2013). Therefore, starch and sucrose contents are among the main indexes of tobacco leaf quality (Dong et al. 2015).

Previous studies showed that genes encoding starch biosynthesis were significantly changed under conditions of drought and heat stress, resulting in decreased starch contents (Yi et al., 2014; Cao et al., 2015). In the present study, we detected 14 DEGs involved in the starch and sucrose metabolism pathway (Table5). These genes are all related to starch and sucrose synthesis or degradation and are vital for plant development. Therefore, changes in their expression levels may indicate differences in starch and sucrose accumulation across different tobacco cultivation regions

Table 1. Number of significantly differentially expressed genes (DEGs) between different cultivation regions.

	Total	Up-regulated	Percent (%)	Down-regulated	Percent (%)
Xingwen vs Miyi	1633	837	51.26	796	48.74
Jiange vs Miyi	2122	1202	56.64	920	43.36
Huili vs Miyi	1399	692	49.46	707	50.54

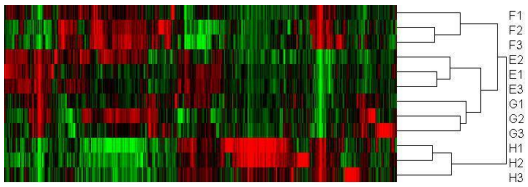

Fig 1. Cluster analysis of tobacco leaf gene expression profiles in four regions. E1-E3 refers to samples from Xingwen, F1-F3 refers to samples from Jiange, G1-G3 refers to samples from Huili and H1-H3 refers to samples from Miyi. Red indicates up-regulated genes and green indicates down-regulated genes.

Table 2. Differentially expressed genes enriched pathways.

Pathway Name	Xingwen vs Miyi		Jiange vs Miyi		Huili vs Miyi	
	P-value	Percent	*P*-value	Percent	*P*-value	Percent
Metabolic pathways	0.006	6.60%	0	9.79%	8.00E-04	6.21%
Endocytosis			0.0023	18.18%	0.0099	12.12%
Thiamine metabolism	0.0096	42.86%			0.0064	42.86%
Plant-pathogen interaction			0.0013	14.49%		
Amino sugar and nucleotide sugar metabolism			5.00E-04	17.98%		
Biosynthesis of phenylpropanoids			0.0025	11.74%		
Starch and sucrose metabolism	0.0094	11.32%	0.0026	15.09%		
Flavonoid biosynthesis			0.003	31.58%		
Phenylpropanoid biosynthesis			0.0055	14.29%		
Glycerolipid metabolism			0.0058	23.33%		
Photosynthesis - antenna proteins					0	42.11%
Photosynthesis	0.0058	13.51%			6.00E-04	14.86%

Note: $P \leq 0.05$

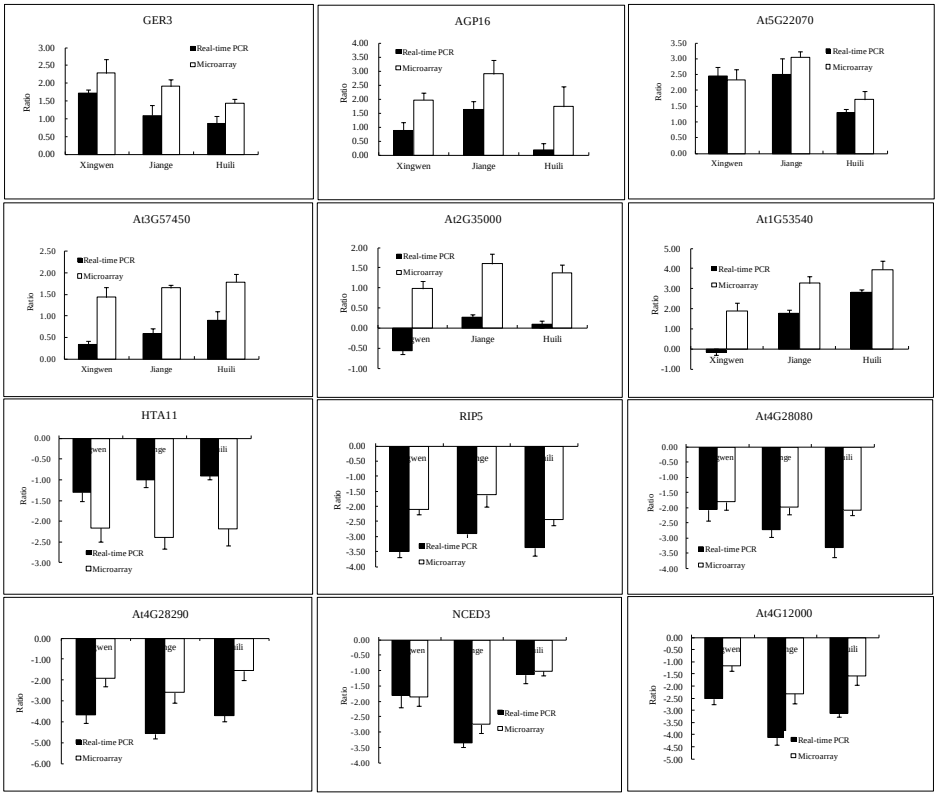

Fig 2. Verification of genes expression patterns by real-time PCR. Ratios refers to the comparison of selected gene expression levels at the regions indicated to Miyi. *AGP16* refers to ADP glucose pyrophosphorylase 16, *GER3* refers to germin-like protein3, *HTA11* refers to Helanthustuberosus agglutinin 11, *RIP 5* refers to ribosome inactivating protein 5, *NCED3* refers to 9-cis-epoxycarotenoid dioxygenase 3. The black box indicates expression patterns detected by real-time PCR, and white box indicates expression patterns detected by microarray.

Table3. The differently expressed genes of antenna protein.

Symbol	Xingwen vs Miyi		Jiange vs Miyi		Huili vs Miyi		Description
	FC	*P* -value	FC	*P*- value	FC	*P*-value	
CAB1	-1.234	0.024			-2.225	0.016	Chlorophyll a-b binding protein 1
LHB1B2	-1.694	0.004			-1.733	0.000	Light-harvesting complex II chlorophyll a/b binding protein 1
LHCA2					-1.062	0.007	Photosystem I light harvesting complex protein
LHCA4					-1.142	0.041	Photosystem I light harvesting complex protein
LHCB3	-1.019	0.011			-1.072	0.009	Light-harvesting chlorophyll B-binding protein 3
LHCB5					-1.383	0.015	Light-harvesting chlorophyll B-binding protein 5

Note: FC, fold change; $P \leq 0.05$

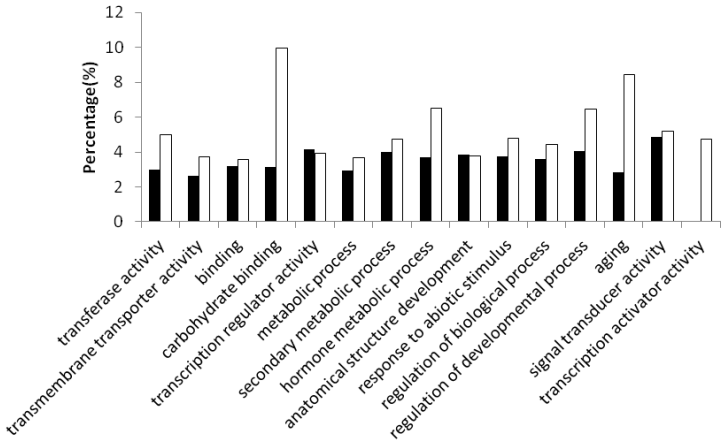

Fig 3. Functional classification of differentially expressed genes. Black box indicates the percentage of up-regulated genes and white box indicates that of down-regulated genes.

during the growth and development of flue-cured tobacco and may result in differences in tobacco leaf quality among the four regions investigated herein.

DEGs involved in ion transportation across membranes

Mineral elements are important for plant growth and development. Many of these elements, such as nitrogen, phosphorus, potassium, sulfur, calcium, magnesium and other trace elements, such as iron, manganese, zinc, boron, chlorine, molybdenum, are essential to tobacco quality and output formation. Some of these mineral elements are required in particular districts (Huang et al., 1999). In the present study, we successfully detected 24 DEGs involved in mineral transportation, of which six DEGs were related to potassium transportation (Table 6). The potassium content of tobacco leaf is significantly related to tobacco leaf quality (Wang and Ma, 2013; Hou et al., 2015; Tang et al., 2015). Lower expression levels of KUP3, KUP9 and SKOR in leaves from Jiange may result in lower tobacco leaf potassium content. Differences in these DEGs and resulting potassium differences may underlie the tobacco leaf quality differences observed among the four areas investigated herein. Compared to the Miyi region, the following DEGs were down regulated more than two-fold: *CAX1*, *CAX3*, *ZIP1* and *ZIP4*. *ZIP4* was the only gene shared by all three comparison groups. Zinc is also a tobacco quality-determining mineral element. According to Huang et al. (1999), zinc is particularly important with regard to tobacco leaf quality. In the present study, *ZIP1* and *ZIP4* were down-regulated compared to the Miyi region suggesting that down-regulation of these DEGs, particularly zinc transporters, may account for the lower leaf qualities observed for these three areas compared to the Miyi region.

Materials and Methods

Plant materials and field experiments

The "Yunyan 87" tobacco (*Nicotiana tabacum* L.) variety employed in this study was cultivated in the following four regions in Sichuan Province, China: Jiange, Xingwen, Miyi, and Huili. Miyi is located in the south of Sichuan Province. The distance from Jiange, Xingwen, and Huili to Miyi is 903, 665 and 96.2 kilometers, respectively. Weather conditions of this four regions can be found in Table7.

In each of the four regions, a 600-square-meter field was selected for study. Plots consisted of 16 rows with lengths of 30 m, generally 120-cm widths and 50 cm between tobacco plants. The sowing date (for tobacco seeds) and transplanting date (for tobacco seedlings) were January 18 and May 1, 2012, respectively. The farmers were responsible for field management practices, including sowing, transplanting, weeding and fertilization. Fertilizer containing 6 kg N, 8 kg P_2O_5 and 15 kg K_2O was manually applied to each plot after transplanting. Tobacco leaf samples were prepared using the middle leaves of the 12^{th} leaf position 70 days after transplanting. From each field, 30 tobacco plants were selected randomly, the leaves of which were cut and frozen in liquid nitrogen for RNA extraction.

RNA isolation and hybridization to gene chips

TRIzol reagent (Invitrogen, Carlsbad, USA) was used for total RNA extraction of tobacco leaf samples. A NanoDrop 2000 fluorospectrometer and formaldehyde denaturing gel electrophoresis were employed to assess the quality of the RNA extracted. An RNeasy Minikit (Qiagen, Valencia, USA) was used for total RNA purification, the product of which was used for cDNA and cRNA generation. An Agilent standard hybridization procedure was used for cRNA

Table 4. The differently expressed genes of carbon utilization.

Symbol	FC	*P* -value	Description
BCA3	-1.19	0.0061	Beta carbonic anhydrase 3
PPC3	-1.04	0.0454	Phosphoenolpyruvate carboxylase 3
SBPase	2.02	0.0001	Sedoheptulose-1,7-bisphosphatase

Note: FC, fold change; $P \leq 0.05$

Table 5. The differently expressed genes of starch and sucrose metabolism.

Symbol	Xingwen vs Miyi		Jiange vs Miyi		Huili vs Miyi		Description
	FC	*P*-value	FC	*P*-value	FC	*P*-value	
ADG1	1.94	0.009	2.71	0.003			Glucose-1-phosphate adenylyltransferase small subunit
ATTPS7			1.22	0.017			Putative alpha,alpha-trehalose-phosphate synthase
BGLU12	-1.14	0.042					Beta glucosidase 12
BGLU15			-2.22	0.004			Beta glucosidase 15
DPE2	-2.23	0.012	-2.99	0.005	-2.52	0.016	4-alpha-glucanotransferase
GAUT12	2.97	0.001					alpha-1,4-galacturonosyltransferase
GAUT13	2.43	0.027	2.09	0.046			Alpha-1,4-galacturonosyltransferase
GAUT14			1.17	0.012			Alpha-1,4-galacturonosyltransferase
HXK1	-1.45	0.018	-1.22	0.007			Hexokinase 1
PGM	-1.01	0.045	-2.16	0.005	-1.46	0.023	Phosphoglucomutase
PHS2	-1.61	0.000	-1.82	0.000	-1.51	0.009	Prostaglandin H synthase-2
SSI1					-1.19	0.032	Soluble starch synthase
SUS3					1.01	0.004	Sucrose synthase 3
TRE1	1.30	0.026					Trehalase 1

Note: FC, fold change; $P \leq 0.05$

Table 6. The differently expressed genes of mineral elements.

Symbol	Xingwen vs Miyi		Jiange vs Miyi		Huili vs Miyi		Description
	FC	*P*-value	FC	*P* -value	FC	*P* -value	
AAT1			-1.23	0.014			Amino acid transporter 1
AMT2			1.25	0.006	1.07	0.049	Ammonium transporter 2
NAXT1			1.10	0.034			Nitrate excretion transporter1
NRT1.7			-1.00	0.020	-1.26	0.013	Nitrate transporter 1.7
PHT4;5			-1.15	0.019			Putative inorganic phosphate transporter 4;5
HKT1	1.17	0.033	1.40	0.010			Sodium transporter HKT1
KUP11	-1.10	0.031					Potassium transporter 11
KUP3			1.11	0.009	1.18	0.007	Potassium transporter 3
KUP5	1.04	0.001					Potassium transporter 5
KUP9	-1.35	0.022	-1.83	0.012			K+ uptake permease 9
SKOR			-1.51	0.037			Potassium channel SKOR
CAX1	-2.51	0.020					Vacuolar cation/proton exchanger 1
CAX3	-2.53	0.015					Vacuolar cation/proton exchanger 3
CAX5			1.38	0.047			Vacuolar cation/proton exchanger 5
CAX9			1.32	0.040			Cation/calcium exchanger 9
SULTR2;1	1.16	0.006			1.68	0.007	Sulfate transporter 2.1
SULTR1;1	-1.69	0.006			1.14	0.001	Sulfate transporter 1.1
ZIP4	-2.09	0.021	-2.08	0.009	-1.61	0.024	Zinc transporter 4 precursor
ZIP1	-1.63	0.017			-2.17	0.010	Zinc transporter 1
ZIP6					-1.10	0.026	Zinc transporter 6
BOR4			-1.59	0.007			Boron transporter 4
CLC-D	-1.221	0.002					Chloride channel protein CLC-d
CCC1			1.29	0.025			Cation-chloride co-transporter 1
MOT1			-1.16	0.005			Molybdate transporter 1

Note: FC, fold change; $P \leq 0.05$

Table 7. Main weather indexes of the four regions.

	Xingwen	Jiange	Huili	Miyi
Annual average temperature (℃)	17.4	15.4	17	19.7
Annual average rainfall (mm)	1234.7	1039.4	1000	1112.6
Annual average humidity (%)	66	69.1	69	65
Annual sunshine (hr)	1010.3	1328.3	2400	2379.3

labeling, fragmenting and hybridization to Agilent tobacco gene chips. The standard wash protocol was used, and a gene chip scanner (Agilent Microarray Scanner, Santa Clara, USA) was utilized for arrays scanning.

Microarray data processing and analysis

Feature Extraction software 10.7 and Gene Spring software 11.5.1 (Agilent Technologies, Santa Clara, USA) were used for raw data collection and normalization, respectively. A gene was selected for expression when it was detected in all three replicates and the *P*-value was less than 0.05. Gene expression levels from Miyi group were used as control, and that from Jiange, Xingwen and Huili were compared to Miyi group respectively, forming three comparing group, Jiange *vs* Miyi; Xingwen *vs* Miyi and Huili *vs* Miyi. The criteria for determining a differentially expressed gene (DEG) were that its expression level changed more than two-fold and that the corresponding *P*-value was less than 0.05. Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used for hierarchical cluster analysis, and differentially expressed genes (DEGs) were classified into functional categories using the gene ontology (GO) web service (http://www.geneontology.org).

qRT- PCR analysis

qRT-PCR analysis was used to confirm the reliability and repeatability of the microarray data. Toward this end, total RNA contents of tobacco leaf samples were extracted as described above, and DNaseI (RNase-free) (Takara, Dalian, China) was applied to eliminate the genomic DNA. Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm) was used to design the gene specific primers utilized in this study. The gene used for normalisation of qRT-PCR data is *Actin*. The procedures for cDNA generation and qRT-PCR were conducted according to Ma et al. (2012).

Conclusions

In general agricultural practice, tobacco producers consider leaf quality more important than yield. Tobacco leaf qualities differ significantly based on the cultivation area. To understand the molecular-level mechanisms that determine tobacco leaf quality, we analyzed the gene expression profiles of tobacco leaves obtained from four tobacco cultivation areas and found that tobacco leaves collected from different planting areas exhibited significant variations in gene expression profiles. Up to 5154 differentially expressed genes (DEGs) were detected among the four areas. These DEGs were classified into 15 categories in relation to transferase activity, transmembrane transporter activity, etc., and were enriched in 11 pathways involved in primary and secondary metabolism, such as photosynthesis, starch and sucrose metabolism, carotenoid and flavonoid biosynthesis, etc. The results reported herein suggest that primary metabolism and secondary metabolism in the tobacco leaf play key roles in tobacco leaf quality.

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