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Integrated analyses of the transcriptome and metabolome of the leaves of albino tea cultivars reveal coordinated regulation of the carbon and nitrogen metabolism

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ABSTRACT

Previous studies have revealed that dramatic changes in metabolism occur in albino tea leaves compared with regular green tea leaves. In particular, the flavonoid content decreased while the amino acid content increased notably, indicating that both the carbon and nitrogen metabolism had undergone significant changes. To understand the coordinated regulation of carbon and nitrogen metabolism in albino tea leaves, we performed integrated analyses of the metabolome and transcriptome for both the albino (*baiye 1*) tea plant and the regular (*Longjing 43* as control) tea plant. Based on the multivariate statistics of principal component analysis and OPLS-DA (orthogonal partial least squares-discriminant analysis), leaves from the *baiye 1* and *Longjing 43* tea plants were classified into three developmental stages. By identifying the main biomarkers from the different groups and from pathway analysis, we gained new insight into understanding the mechanism of the carbon and nitrogen metabolism in the leaves of the albino mutant plants: the serious weakening of the carbon metabolism in the albino leaves led to a reduced nitrogen consumption. However, nitrogen catabolism was enhanced to generate/supply more carbon skeletons for energy metabolism, which helped to coordinate the dramatic changes in metabolism resulting from the carbon-deficiency stress in the albino leaves. Moreover, the reallocation of carbon and highly efficient recycling of endogenous ammonium also constituted a potential mechanism for regulating the balance of carbon and nitrogen metabolism in albino leaves under carbon-deficiency stress. All of these metabolic responses in albino leaves create a potential mechanism for regulating the balance of carbon and nitrogen metabolism under conditions of carbon deficiency. Moreover, the coordinated regulation of the carbon and nitrogen metabolism, including reallocation of carbon resource from secondary metabolites and amino acids, highly efficient way to recycle and store endogenous ammonium, plays a great role for the albino tea plant in surviving from the carbon deficiency stress.

1. Introduction

Carbon (C) metabolism and nitrogen (N) metabolism are the two main metabolic processes occurring in plants. Both processes need to assimilate the organic carbon and energy produced during photosynthesis. Furthermore, this requirement may be responsible for the negative correlation between yield and quality in plants (Ruan et al., 2010). Moreover, there is continuous interaction between carbon and nitrogen metabolism (Oliveira and Coruzzi, 1999; Ruan et al., 2010), and this interaction varies from species to species (Oliveira and Coruzzi, 1999). For tea plants, amino acids, especially phenylalanine, are the main upstream product of flavonoid metabolism (the main quality component in tea) (Kito et al., 1968), and our previous study suggested that the supply of nitrogen also has a significant effect on the

Abbreviations: PCA, principal component analysis; OPLS-DA, orthogonal partial least squares-discriminant analysis; C, carbon; N, nitrogen; TEM, transmission electron microscopy; GC??GC-ToF-MS, two-dimensional gas chromatography-time-of-flight mass spectrometry; UPLC-Q-TOF/MS, ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry; VIP, variable importance in projection; HPLC-DAD, high-performance liquid chromatography with diode-array detection; KEGG, Kyoto Encyclopedia of Genes and Genomes; EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate; ECG, epicatechin gallate; GCG, gallocatechin gallate; DEGs, differentially expressed genes; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GS/GOGAT, GS/glutamine oxoglutarate aminotransferase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4Cl, 4-coumarate–CoA ligase; CHI, chalcone isomerase; CHS, chalcone synthase; F3???5???H, flavonoid 3???, 5???-hydroxylase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-monooxygenase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase

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proportion of carbon and nitrogen in tea (Ruan et al., 2010). Likewise, both the carbon- and nitrogen-containing metabolites could be affected by genotype, nutrition and environmental factors (Harbowy et al., 1997; Ruan et al., 2010; Wang et al., 2014). Liu et al. (2016) have shown that the rate of photosynthesis increases continuously with increasing temperature and light intensity while the rate of nitrogen metabolism and accumulation of sugar, starch and flavonoids decreases (Liu et al., 2016). However, little information about the mechanism of interaction or relationship between the C metabolism and N metabolism of tea plants has been reported. Thus, our study on the coordinated regulation of the C and N metabolism in tea plants can help to improve the tea quality, and also to regulate the balance between yield and quality.

Unlike animals, plants are unable to escape from adverse circumstances. To cope with external stresses, plants modulate their metabolic systems, especially the secondary metabolism, which results in an accumulation of specific metabolites (Satou et al., 2014) such as catechins and flavones (Agati et al., 2013). By contrast, in starvation, cells capture their own cytoplasm and organelles and consume them in lysosomes (Rabinowitz and White, 2010); for example, the autophagic degradation of chloroplasts is particularly activated in leaves under sugar-depleted conditions (Masanori et al., 2013). Benefiting from such responses, plants have survived and built up a new C/N metabolic balance. For example, the albino mutant plants attempt to activate their C metabolism in order to gain energy, reclaim chloroplast functions and protect against environmental stress (Satou et al., 2014).

Albino tea leaves have been reported to show enhanced levels of free amino acids and low polyphenol content, which improves the quality of made tea, and impart to it a higher economic value than non-albino varieties (Ma et al., 2012; Feng et al., 2014). *Baiye 1*, a temperature-sensitive chlorophyll-deficit mutation variety (Ma et al., 2012), has been accepted as one of the most popular cultivars for green tea. Microarray methods (Ma et al., 2012), proteomic analysis (Li et al., 2011), enzyme analysis (Xiong et al., 2013) and structural analysis of chlorophyll (Du et al., 2008) have all been used to study the correlation between phenotype and the principal quality components of *baiye 1* (Xiong et al., 2013; Feng et al., 2014). However, a systematic approach has seldom been used to study the relationship between C and N metabolism in albino tea plants. This study was designed to explore the coordinating mechanism of C and N metabolism in albino plants using integrated analyses of the transcriptome and metabolome of the leaves.

2. Materials and methods

2.1. Plant material and growth conditions

14-year-old tea cultivar ???*baiye 1*??? and ???*Longjing 43*??? was grown in the China National Germplasm Hangzhou Tea Repository located at the Tea Research Institute of the Chinese Academy of Agricultural Sciences (latitude: N 29.93, longitude: E 120.69). The tea bushes were planted in double rows with an inner row distance of 40 cm, an outer row distance of 140 cm, and a 33-cm space between bushes within each row. The owner of the plantation fertilized the plants with N, P_2O_5 and K_2O at levels of 600, 300 and 300 kg ha$^{????1}$ per year, respectively, to provide adequate nutrients. Between April 1 and June 13, the second leaves of young shoots were collected at 10 time points, with sampling intervals of 4???7 days. Three time points (begin, middle and end) has been chosen to collect the sample of regular species (*Longjign 43*) fit to the phenotype of *baiye 1*. The harvested samples were frozen with liquid nitrogen immediately and then placed in a refrigerator at ????70 ??C. Sampling was biologically repeated six times from different individual plants. Freeze-dried samples were also pulverized by a ball-miller (M301 Retsch, Germany) so that the total content of C and N could be analysed. Climatic parameters were automatically recorded in a meteorological station close to the experimental field (Table S1).

2.2. Electron microscopic analysis

Transmission electron microscopy (TEM) was used to observe the ultrastructure of the albino leaf. Leaf samples (about 1 mm^2) were fixed with 2.5% glutaraldehyde solution overnight at 4 ??C, the ultra-thin sections were cut and stained, and viewed under a JEM-1230 transmission electron microscope at an accelerating voltage of 80 kV, as described by Li et al. (2016).

2.3. Metabolome analysis using gas chromatography???mass spectrometry

The tea leaves (100 mg) were extracted with 1000 ??L of methanol-chloroform (3:1, v/v) solvent. The extraction was then mixed with 10 ??L of L-2-chlorophenylalanine (0.3 mg/mL in water). The samples were centrifuged for 10 min at 12,000 rpm at ???4 ??C; the supernatant (400 ??L) was first dried in a vacuum concentrator without heating and the dried completely under nitrogen. We added 80 ??L methoxyamine (15 mg/mL in pyridine) to the dried sample and vortexed it for 1 min. Methoxymation was performed at 37 ??C for 120 min. Subsequently, the sample was trimethylsilylated at 70 ??C for 90 min by adding 80 ??L BSTFA (N, O-Bistrifluoroacetamide) containing 1% TMCS (trimethylchlorosilane) to the solution (Lisec et al., 2006; Liu et al., 2015).

Two-dimensional gas chromatography-time-of-flight mass spectrometry (GC ?? GC???ToF-MS) was performed using an Agilent GC 6890N gas chromatograph with a dual-stage cryogenic jet modulator (licensed from Zoex) and a secondary oven. The detector was a high-speed ToF mass spectrometer (Pegasus HT, Leco Co., CA, USA). Each 1 ??L aliquot of the derivatized sample was injected in splitless mode into the GC ?? GC???ToF mass spectrometer. A DB-5 MS capillary column (30 m ?? 250 ??m i.d., 0.25 ??m film thickness; J&W Scientific, Folsom, CA, USA) was used as the first-dimension column and a DB-17H 2.5 m ?? 0.1 mm I.D., of 0.1-??m film thickness, was used as a second-dimension column. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was set at 280 ??C. GC initial column temperature was set at 90 ??C for 2 min and increased to: 180 ??C at a rate of 8 ??C/min, 240 ??C at a rate of 3 ??C/min, and then 290 ??C at a rate of 25 ??C/min. Finally, it was kept at 290 ??C for 18.25 min. The secondary oven temperature was kept at 5 ??C, offset above the primary oven. The transfer line temperature and ion source temperature were set at 270 ??C and 220 ??C, respectively. The modulation time was 6 s; the modulator temperature was kept at 15 ??C, offset above the secondary oven. Electron impact ionization (70 eV) at full scan mode (m/z 30-600) was used to acquire mass spectra. The dwell time for each scan was set at a rate of 50 spectra per second, and the solvent delay at 5 min.

Total ion chromatograms were processed using the automated data processing software ChromaTOF (v3.30, Leco Co., CA, USA) at a signal-to-noise threshold of 500 dB. Metabolite identification from these selected variables was achieved by referring to NIST 05 Standard mass spectral databases (NIST, Gaithersburg, MD, USA). The resulting data???containing sample information, peak retention time and peak intensities???were normalized to the area of the internal standard (internal standard peaks were removed afterward), and then mean-centered. Univariate statistics were performed using the method used in UPLC-Q???TOF/MS (ultra-performance liquid chromatography quadrupole ToF-MS) metabolome analysis.

2.4. Metabolome analysis using UPLC-Q-TOF-MS

The metabolites in a sample of young shoots were extracted with 75% methanol and 1% formic acid, as described by Zhang et al. (2014). A 2 ??L sample was injected in UPLC-Q-TOF/MS (Waters, UPLC/Xevo G2-S Q-TOF) and separated with an HSS T3 column as described by Zhang et al. (2014).

Data preprocessing was performed using TransOmics software. Metabolite peaks were assigned from the accurate mass measurements